Journal of Carcinogenesis
Covering Genesis to Therapeutics

Journal of Carcinogenesis, ISSN: 1477-3163
2025; Vol 24: Issue 10s, https://carcinogenesis.com/

Host-Microbe Metabolite Interactions in Asymptomatic C. Difficile Carriers: A Combined qPCR and SCFA Profiling Study.

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ABSTRACT

Background: Asymptomatic colonization with *Clostridioides difficile* (C. difficile) is common, yet the microbial and metabolic dynamics that distinguish silent carriage from overt infection remain poorly understood. Short-chain fatty acids (SCFAs) and calprotectin may serve as functional biomarkers reflective of gut homeostasis and inflammation.

Objective: This study aimed to evaluate host-microbe metabolic interactions in asymptomatic *C. difficile* carriers through combined quantitative PCR (qPCR), gas chromatography, and enzyme immunoassay analysis, with a focus on toxin gene loads, SCFA concentrations, and fecal calprotectin.

Methods: In this cross-sectional study, 50 adult participants were recruited—25 asymptomatic carriers and 25 healthy controls. Stool samples underwent qPCR to quantify total bacterial load and the toxin genes *tcdA* and *tcdB*. SCFA levels (acetate, propionate, butyrate) were measured via gas chromatography, while fecal calprotectin was assessed using ELISA. Statistical analysis included t-tests, chi-square tests, logistic regression, and multivariate linear regression.

Results: Toxin gene loads (*tcdA*, *tcdB*) were significantly higher in carriers compared to controls ($p < 0.0001$), whereas total bacterial load showed no significant difference ($p = 0.38$). SCFA concentrations were significantly reduced in carriers: acetate (50.6 vs. 60.6 mmol/kg), propionate (17.8 vs. 22.2 mmol/kg), and butyrate (11.1 vs. 16.1 mmol/kg); all $p < 0.0001$. Fecal calprotectin levels were significantly elevated in carriers (102.8 vs. 44.3 mg/kg, $p < 0.0001$). Logistic regression identified BMI, calprotectin, and lower butyrate and acetate as independent predictors of carriage. Multivariate analysis revealed *tcdA* load as the primary predictor of calprotectin elevation.

Conclusion: Asymptomatic *C. difficile* carriage is characterized by elevated toxin gene loads, reduced SCFA levels, and subclinical inflammation. These findings support a metabolic-inflammatory axis in asymptomatic colonization and suggest that integrating microbial, metabolic, and immune markers can improve risk stratification in C. difficile surveillance..

How to Cite: Wasan Riyadh Mohammed, Wahran Khudhair Abbas, Amidah.Ali.Atiyah , Asmaa Easa Mahmood, (2025) Host-Microbe Metabolite Interactions in Asymptomatic C. Difficile Carriers: A Combined qPCR and SCFA Profiling Study..., *Journal of Carcinogenesis*, *Vol.24*, *No.10s*, 317-331.

1. INTRODUCTION

Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are essential metabolites produced by gut microbiota through fermentation of dietary fibers and are key to gut health and immune function (1). These SCFAs maintain intestinal barrier integrity by nourishing colonocytes and enhancing tight junction protein expression (2). Butyrate, in particular, promotes anti-inflammatory responses by inducing regulatory T cells and inhibiting histone deacetylases (4). SCFAs also signal through G-protein coupled receptors to modulate epithelial and immune cell responses, aiding in the clearance of pathogens and preventing overactive inflammation (4). Calprotectin, a calcium-binding antimicrobial protein, serves as a marker of intestinal inflammation and is influenced by SCFA levels and microbiota composition (5). Disruptions in SCFA production and elevated calprotectin are linked to inflammatory bowel diseases, underscoring their combined role in mucosal defense and immune regulation (6).

Asymptomatic colonization with *Clostridioides difficile* is common across populations, with studies showing prevalence

rates as high as 48% in transplant patients and 14% in children with inflammatory bowel disease (7), (8). Approximately 3–5% of asymptomatically colonized patients develop infection, especially in the presence of risk factors like recent antibiotic use or immunosuppression (9), (10). Altered gut microbiota with elevated short-chain fatty acid (SCFA)-producing bacteria has been associated with stable colonization without progression to disease, suggesting metabolic adaptations may influence outcomes (11).

Fecal calprotectin and IL-1β levels, although elevated in active *C. difficile* infection, do not rise in asymptomatic carriers, indicating a lack of significant inflammation in colonized individuals (12), 13). These findings support the rationale for investigating SCFAs and calprotectin as biomarkers to understand metabolic and inflammatory responses during asymptomatic colonization and its potential shift toward pathogenic states (14).

The objectives of this study were grounded in the growing need to understand the early molecular and metabolic events that precede symptomatic Clostridioides difficile infection. While it had been well documented that C. difficile could colonize the gastrointestinal tract in the absence of clinical symptoms, the mechanisms underlying this silent carriage and the factors that might precipitate a transition to active disease remained unclear. Therefore, the study sought to explore whether measurable differences in microbial metabolites, particularly short-chain fatty acids (SCFAs), could reflect or even predict changes in C. difficile toxin gene activity.

To achieve this, the study aimed to evaluate the relationship between the load of toxigenic genes—specifically *tcdA* and *tcdB*—and the concentrations of key SCFAs such as acetate, propionate, and butyrate in stool samples from asymptomatic C. difficile carriers. It was hypothesized that fluctuations in SCFA levels might be linked to microbial dynamics that either suppress or facilitate toxin gene expression, thereby offering a window into gut homeostasis at the molecular level. By employing quantitative PCR for gene expression and gas chromatography for metabolite profiling, the research intended to determine whether these biological signals exhibited meaningful correlations.

Another objective was to assess the extent of subclinical inflammation in asymptomatic carriers compared to healthy individuals by quantifying fecal calprotectin levels. This allowed for an additional layer of analysis to determine whether toxin gene presence and metabolic shifts coincided with subtle immunological responses that could otherwise go undetected in standard clinical assessments.

Ultimately, the study endeavored to develop an integrated understanding of host-microbe metabolic interactions in asymptomatic C. difficile colonization. By doing so, it aimed to lay the groundwork for predictive models that could identify individuals at risk of progressing to symptomatic infection, using non-invasive biomarkers instead of imaging-based diagnostics.

2. METHODOLOGY

Study Design and Ethical Approval

The study was a cross-sectional, case-control analysis that attempted to study the host-microbe metabolic interactions in the scenario of asymptomatic Clostridioides difficile colonization. The study adopted a molecular and biochemical methodology to investigate the relationship between the presence of toxigenic genes, metabolites levels, and subclinical inflammation of human stool samples. The cross-sectional design allowed a cross-sectional comparison of two different groups: asymptomatic C. difficile-positive and healthy controls, no longitudinal follow-up or intervention. This design was used to discover measurable biological trends that might reflect preclinical conditions of disease without affecting the natural microbial profile of the participants either as a result of treatment or surveillance.

The study was in a comparative design whereby the two groups were sampled under controlled conditions to achieve uniformity in processing and handling. The selection criteria were strictly used to make sure that the study involved the asymptomatic stage of colonization and that the subjects did not have recent antibiotic therapy, hospitalization, or had symptoms of gastrointestinal disorders. This was done to separate asymptomatic colonization and acute infection and other confounding factors to permit a more detailed investigation of microbial and host derived factors. The study groups have been matched as close as possible to age and sex to ensure that the demographic differences are reduced and help in the internal validity of the results.

Special consideration was given to scientific integrity and ethical rigor of the research. The institutional review board provided ethical consent before any study activities commenced. The study followed all the principles of the declaration of Helsinki and the ethics of the host institution in all the processes that involved human-derived specimens. Ethics committee conducted a formal review of the study aims, methodology and sample handling procedures to determine the risk-benefit profile and the protection of participants confidentiality and well-being.

All participants provided informed consent and the consent procedure involved a clear description of the purpose of the study, the kind of analyses that would be carried out, how the data would be used and what would happen to ensure anonymity and voluntary participation. Emphasis was made on the fact that no clinical intervention was involved in participation and that all the results obtained would be utilized purely on a research basis. The participants were guaranteed

that they could withdraw any time without penalty. Signed consent forms were properly stored as per data protection rules and unique identifiers were applied to ensure anonymity in the sample labeling process and data entry.

The study infrastructure was put in place through partnership with clinical microbiology laboratories who provided the entry point of identification of potential participants and retrieval of eligible samples. The samples were taken in regular clinical operations, and de-identified before they were handed over to the research team. This arrangement reduced interference in the treatment of patients and maintained the originality of the biospecimens by stabilizing and rapidly freezing them immediately after collection.

The general workflow of the study was designed in such a way that it could reduce bias and be reproducible. Molecular and metabolomic assays were carried out by researchers who were blinded of the group assignment of the samples to remove any bias in the interpretation of the data. A study timeline was closely monitored and all sample processing, molecular extractions and analysis were done within consistent time frames to help mitigate temporal variability in storage conditions or reagent performance. In addition, biosafety and molecular diagnostic procedures were offered to all the staff engaged in laboratory work to ensure uniformity in the procedures of handling human biological material.

Participants were not given any financial incentive to prevent excessive enticements and the study was conducted within the budgetary limits allowed by the supporting academic institution. All reagents, consumables and instrumentation employed in the research had been pre-approved to be used in human specimen research and all laboratory equipment had quality assurance logs to ensure that it was within research parameters.

Such ethical and methodological basis enabled the generation of reliable data on the expression of microbial genes, host inflammatory markers, and metabolic products without compromising the rights of participants or creating unneeded sources of variability. Through this rigorous design and monitoring procedures, the study was able to guarantee that the conclusions they made were based on sound, ethical scientific practice.

Participant Selection, Sample Size, and Sample Collection

The participants were identified with the help of a targeted screening process with the diagnostic microbiology laboratories associated with tertiary care hospitals. The inclusion criteria of the study were well-planned to isolate the members who were carriers of Clostridioides difficile and who did not show clinical symptoms. Study group participants had to be adults with positive C. difficile testing during routine stool diagnostic testing with no evidence of diarrhea, abdominal pain, or fever at the time of testing or within the past week. The control group consisted of individuals who were sampled within the general outpatient population who had been tested with stool sample due to reasons other than C. difficile infection and were negative to C. difficile, along with other enteric pathogens.

In order to have a strong and statistically significant comparison, the study employed a balanced sample that is 50 participants. This group was stratified into two arms, 25 asymptomatic C. difficile-positive people who were the study group and 25 healthy and C. difficile-negative people who were the control group. Sample size was calculated using priori power calculations to identify medium effect sizes and a power of 80 percent of key outcome measures including metabolite concentrations and gene expression levels with confidence level of 95 percent. The previous research on the topics of gut metabolomics and microbial gene quantification informed these estimates, and thus the final design was optimized to be both statistically rigorous and logistically feasible.

The study participants were recruited consecutively based on their ability to meet the eligibility criteria and all participants were expected to sign a written informed consent prior to inclusion of their samples in the study. Particular attention was paid to avoiding people with recent antibiotics or immunosuppressive treatment, or a history of chronic gastrointestinal disorders, including inflammatory bowel disease or irritable bowel syndrome, since these conditions might interfere with the microbial and metabolic profiles of interest. Also, patients who had been admitted in the previous 30 days were not considered because it was likely to lead to nosocomial effects on the intestinal microbiota.

After eligibility was established and consent was given, stool samples were obtained as per the standard clinical procedures that had been established in the collaborating institutions. Samples were taken in sterile, leak-proof bottles and processed immediately so that the microbial DNA and volatile metabolites could be maintained. To minimize transient metabolic changes, participants were asked to abstain from probiotic supplements, and radical dietary modifications during 48 hours before donating the samples. All the samples were taken in a specific period of two months to reduce the seasonal or environmental differences that might affect the microbial composition or metabolic production.

On arrival, anonymization of stool samples was done and a study code assigned to retain confidentiality. The samples were visual checked by consistency and the volume, and only specimens with minimum quality and quantity were accepted to the final dataset. Each sample was quickly aliquoted into pre-labelled cryovials that enabled simultaneous analysis of DNA, metabolites and inflammatory markers without subjecting them to repetitive freeze-thaw cycles. Aliquoting was carried out under a controlled environment with pre-chilled instruments and equipment in order to preserve the integrity of the molecules.

During the collection process, all samples were flash-frozen in dry ice or liquid nitrogen within one hour and then maintained at -80 o C till further processing. The transportation of the samples between collection points and the central laboratory was conducted in validated cold-chain systems that had continuous monitoring of temperature to meet the standards of biospecimen handling. Each sample was kept in chain-of-custody records, including time points, the personnel who handled the sample, and storage conditions.

The consistency in sample handling protocols across both study arms minimized potential pre-analytical biases and enabled high comparability between groups. This rigorous framework for participant selection and sample collection ensured that the resulting dataset accurately reflected the underlying biological state of the gut environment in asymptomatic *C. difficile* carriers compared to healthy controls. By controlling for extraneous variables at this foundational stage, the study established a reliable basis for subsequent molecular and biochemical analyses aimed at elucidating host-microbe interactions.

DNA Extraction and Quantitative PCR Analysis

Genomic DNA was isolated from stool aliquots using the QIAamp Fast DNA Stool Mini Kit (Qiagen, catalog no. 51604), following the manufacturer's streamlined spin-column protocol designed for rapid removal of PCR inhibitors. Each extraction utilized 180–220 mg of frozen stool, homogenized in InhibitEX Buffer and incubated with Proteinase K at 70 °C. Subsequent silica-membrane binding and wash steps—performed at 20,000 × g—ensured purification of high-quality bacterial and human DNA. Elution in 200 µL of Buffer AE yielded typical DNA concentrations of 5–50 µg, sufficient for multiple downstream assays . To verify DNA integrity and purity, A260/280 and A260/230 ratios were measured using a NanoDrop spectrophotometer; acceptable values were between 1.8–2.0 and ≥1.8, respectively, with concentrations adjusted to a final working range of 10–20 ng/µL.

Quantitative real-time PCR assays were conducted to quantify total bacterial load (via 16S rRNA gene) and the toxigenic loci *tcdA* and *tcdB*. Custom TaqMan-based primer and probe sets targeting conserved regions were employed, with specificity confirmed by >95 % homology across reference *C. difficile* genomes. Each 25 µL reaction contained 12.5 µL of lyophilized Tetra™ MasterMix (YouSeq, resuspended per manufacturer's 1.5 mL buffer), forward and reverse primers at 400 nM each, a 200 nM fluorescent probe, 5 µL of template DNA, and was completed with nuclease-free water. Amplifications were performed on a Bio-Rad CFX96 real-time PCR system under the following thermal cycling: initial denaturation at 95 °C for 2 minutes, followed by 45 cycles of 95 °C for 15 seconds and 58 °C for 45 seconds, with fluorescence acquisition during the annealing phase.

Each qPCR plate incorporated a no-template control, a positive control (purified *C. difficile* genomic DNA), and an internal amplification control targeting a housekeeping gene to monitor inhibition. The analytical sensitivity reached approximately 0.45 copies/µL for both *tcdA* and *tcdB* targets, as determined by Probit analysis. Standard curves were generated from ten-fold serial dilutions of quantified *C. difficile* ATCC DNA to calculate absolute gene copy numbers per gram of stool. Amplification efficiency consistently ranged between 90–110 %, with R^2 values exceeding 0.98, indicating reliable performance across detection ranges.

Total bacterial load was quantified by amplification of the universal bacterial 16S rRNA gene, using primers and probe sequences adapted from Kubota et al. (15). These reactions matched the thermal profile used for toxigenic gene assays, with separate standard curves developed from *Escherichia coli* genomic DNA to convert Ct values into copy numbers. All qPCR assays were performed in triplicate to enhance accuracy and reproducibility. Mean Ct values and standard deviations were calculated, and runs with triplicate variation exceeding 0.5 Ct were flagged and rerun to maintain precision.

Normalized expression values for *tcdA* and *tcdB* were derived using the ΔCt method against 16S rRNA reference levels. Absolute quantification also yielded copy-number ratios (toxigenic gene copies per total bacterial 16S copies and per gram of stool). Data were stored in a secure laboratory database, with sample identifiers coded to maintain blinding with respect to asymptomatic carriers and control status. Quality control metrics—including DNA yield, purity, Ct values, amplification efficiency, and triplicate consistency—were reviewed at the conclusion of each batch. Any sample failing quality thresholds (e.g., Ct > 35 in positive controls or inconsistent amplification curves) was re-extracted and re-analyzed.

This methodology enabled robust and precise quantification of both overall bacterial burden and targeted toxin gene abundance, setting the foundation for subsequent correlation with metabolomic and inflammatory markers in downstream analyses.

SCFA Extraction and Gas Chromatography Protocol

Stool aliquots designated for metabolite analysis were thawed on ice and homogenized in 5 mL of ice-cold, LC-MS-grade water. The homogenate was acidified by adding 50 µL of concentrated hydrochloric acid to achieve a pH of approximately 2.0, ensuring the protonation of free fatty acids in preparation for efficient extraction. The acidified suspension was centrifuged at 10,000 × g for 10 minutes at 4 °C to pellet solids, and the supernatant was transferred to a fresh amber vial for the extraction step.

Short-chain fatty acids were isolated by liquid–liquid extraction using 2 mL of methyl tert-butyl ether (MTBE, HPLC grade, Sigma-Aldrich). This solvent was selected over ethyl acetate due to lower background noise and superior recovery profiles. The mixture was vortexed for 1 minute and then centrifuged at 4,000 × g for 5 minutes. The organic phase was carefully decanted into a new vial and evaporated under a gentle stream of nitrogen at 30 °C until dryness, typically requiring 15–20 minutes.

The dried extract was reconstituted in 200 µL of MTBE containing an internal standard mixture of ^13C-labeled SCFAs (acetate-^13C_2, propionate-^13C_3, butyrate-^13C_4; Cambridge Isotope Laboratories) at a final concentration of 10 µM each. This preparation enabled accurate quantification and correction for extraction variability. After another brief vortex, samples were transferred to 200-µL GC autosampler vials equipped with glass inserts.

Chromatographic separation was conducted using an Agilent 7890B gas chromatograph coupled with a 5977B mass spectrometer and equipped with a DB-FFAP column (30 m × 0.25 mm × 0.25 µm). Injection volume was 1 µL in splitless mode, with the injector temperature set to 230 °C. The oven temperature program began at 80 °C (held for 1 minute), ramped at 10 °C/minute up to 200 °C, and held for 5 minutes. Transfer line, MS quadrupole, and ion source temperatures were maintained at 250 °C, 150 °C, and 230 °C, respectively. Ionization was performed in electron impact mode at 70 eV, with data acquisition acquired in selected ion monitoring (SIM) mode to enhance sensitivity for ions specific to acetate (m/z 60), propionate (m/z 74), and butyrate (m/z 88).

Calibration curves were constructed using serial dilutions of SCFA standards (Sigma-Aldrich, catalog no. SBR00030) covering 0.1 to 20 mM, reflecting physiological fecal concentrations. These standards underwent the identical extraction and derivatization process as the study samples. Linearity was validated with R^2 values greater than 0.995 for all analytes. Limit of detection (LOD) ranged from 0.05 to 0.1 mM, and limit of quantification (LOQ) ranged from 0.1 to 0.2 mM, in line with published fecal SCFA analytical quality benchmarks.

Each batch included one blank control, one quality control made from pooled fecal extract, and five calibration standards. Batch-to-batch consistency was monitored by evaluating retention time drift (<0.05 min) and peak area coefficient of variation (<8%). All extracted ion chromatograms were reviewed using Agilent MassHunter Quant software. Peak integration employed Gaussian smoothing and a peak width of 0.15 min. Absolute SCFA concentrations in stool were back-calculated, taking sample weight and dilution factors into account, and were expressed in mmol per kg wet weight.

This protocol enabled precise, reproducible quantification of acetate, propionate, and butyrate in stool. The selection of MTBE extraction, pH control, targeted MS–SIM acquisition, and rigorous calibration ensured high analytical quality and comparability across samples. It provided metabolic readouts essential for investigating the role of these SCFAs in C. difficile toxigenic expression and subclinical inflammation.

Fecal Calprotectin Quantification by Enzyme Immunoassay

Stool samples underwent calprotectin analysis using a sandwich-style ELISA. Approximately 100 mg of thawed stool had been weighed and extracted in 5 mL of kit-supplied buffer, prepared in accordance with manufacturer instructions. The extraction involved vortexing the mixture vigorously for 30 minutes at room temperature, followed by centrifugation at 3,000 × g for 10 minutes, to separate solid debris from the soluble fraction that contained the target protein. Supernatants were then transferred into fresh tubes for assay.

Calprotectin quantification was conducted using the Immuchrom Calprotectin (S100A8/A9) ELISA Kit (ImmuChrom GmbH, catalog IC7300), a research-use sandwich immunoassay validated for human stool. This kit utilized microtiter wells pre-coated with a monoclonal antibody against calprotectin. Quantitative detection was achieved by sequentially incubating extracted samples and standards with an HRP-conjugated detection antibody. Each sample run included duplicates of a seven-point standard curve with recombinant calprotectin ranging from 3 ng/mL to 208 ng/mL, as well as low and high controls provided by the manufacturer. All reagents were brought to room temperature prior to use to ensure optimal antibody-antigen binding kinetics.

The assay protocol began with the addition of 100 µL of each standard, control, and diluted sample into designated wells. The plate was then incubated at 37 °C for 60 minutes, followed by three washes using a wash buffer to remove unbound material. Subsequently, 100 µL of HRP-labeled detection antibody was applied to each well and the plate incubated again at room temperature for 30 minutes, under gentle shaking. Unbound conjugate was removed through another series of washes. Substrate solution, TMB (3,3',5,5'-tetramethylbenzidine), was then added at 100 µL per well and the plate incubated in the dark for 15 minutes, during which a blue chromogenic reaction developed in wells containing bound calprotectin. The enzymatic reaction was terminated by adding 50 µL of stop solution (1 M phosphoric acid), which also resulted in a color shift from blue to yellow. Optical density readings were recorded at 450 nm with background correction at 620 nm using a BioTek ELx800 microplate spectrophotometer.

The kit's reported limit of detection was 0.997 ng/mL and its calibration curve range extended linearly to 208 ng/mL. Intra-assay variability was below 10 % CV and inter-assay variability remained under 15 % CV. Calibration curves were constructed using four-parameter logistic (4PL) regression via Gen5 software. Sample concentrations were back-calculated

and expressed in mg/kg wet stool. A dilution factor of 1:50 from extraction to final assay volume had been predetermined, yielding an estimated physiological detection range of 10–8000 mg/kg, compatible with clinical ranges reported by DiaSorin and others for severe inflammation labindustrias.com+14fishersci.com+14buhlmannlabs.com+14.

Every batch included a blank well, controls to monitor assay integrity, and pooled stool quality control samples to assess inter-batch consistency. Acceptance criteria required that standard curve R-squared values exceeded 0.995, control concentrations fell within expected ranges, and blank wells measured absorbance ≤0.05. Any sample with duplicate readings differing by more than 15 % CV was re-tested. All reagents, including buffers and conjugates, were stored at 2–8 °C and allowed to equilibrate to room temperature prior to use; kit expiration dates and storage conditions were strictly monitored.

Raw data underwent quality review; outliers resulting from pipetting errors or plate anomalies were flagged. Calprotectin concentrations falling outside the standard curve's dynamic range prompted re-assays using adjusted dilution factors based on preliminary values. Final concentrations were calculated by applying stool weight, extraction volume, and assay dilution corrections, resulting in values expressed as mg of calprotectin per kg of wet stool.

Sample identifiers were de-linked from group assignment to maintain blinding during analysis. All data, including optical densities, calculated concentrations, dilution factors, assay batch numbers, kit lot numbers, operator ID, and date of assay, were logged into a secure laboratory information management system. This structured approach ensured that calprotectin measurements were precise, reproducible, and compatible with clinical and research standards for evaluating subclinical intestinal inflammation.

When analyzed, this systematic quantification of fecal calprotectin allowed the study to compare inflammatory markers between asymptomatic *C. difficile* carriers and healthy controls in a robust and controlled manner, establishing one of the critical immunological endpoints of the investigation.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 28.0 (IBM Corp., Armonk, NY). Data were assessed for normality using the Shapiro–Wilk test. Continuous variables were expressed as mean ± standard deviation and compared between groups using independent samples t-tests. Categorical variables (e.g., gender) were analyzed using Pearson's chi-square test. All significance tests were two-tailed, with a p value < 0.05 considered statistically significant.

To explore relationships between predictor variables and asymptomatic *C. difficile* carriage, a binary logistic regression model was applied. The dependent variable was group status (carrier vs. control), and independent variables included BMI, butyrate, acetate, calprotectin, and age. Odds ratios (ORs) with 95% confidence intervals (CIs) were reported.

To determine independent predictors of fecal calprotectin concentration, a multivariate linear regression model was constructed. The dependent variable was calprotectin (mg/kg), and independent variables included *tcdA* load, *tcdB* load, butyrate, BMI, and total bacterial load. Regression coefficients, standard errors, and 95% CIs were reported. A p value < 0.05 was used to indicate statistical significance across all regression analyses.

Spearman's correlation coefficients were calculated to assess non-parametric associations among SCFAs, toxin gene loads, BMI, and calprotectin levels. Correlation matrices were visualized using a heatmap.

Results

Table 1:Statistical Comparison of Demographic Variables Between Carrier and Control Groups

Variable	Carrier (n = 25)	Control (n = 25)	p value
Age Mean (SD)	58.4 (9.6)	57.1 (9.3)	0.64
BMI Mean (SD)	28.5 (4.4)	25.7 (3.0)	0.013
Male Gender n (%)	11 (44.0%)	15 (60.0%)	0.40

Statistical tests used: Independent samples t-test for continuous variables (Age and BMI). Pearson's chi-square test for categorical variable (Gender). A p value below 0.05 was considered statistically significant.

The analysis demonstrates that the mean age of participants in the carrier group was 58.4 years with a standard deviation of 9.6, while the control group had a similar mean age of 57.1 years and a standard deviation of 9.3. This small difference was not statistically significant, indicating comparable age distribution between the two groups. For BMI, carriers exhibited a higher mean value of 28.5 and standard deviation of 4.4 compared to controls, whose mean BMI was 25.7 with a standard

deviation of 3.0. This difference reached statistical significance with a p value of 0.013, suggesting a noteworthy disparity in BMI between the groups. Regarding gender, males represented 44% of the carrier group and 60% of the control group. The proportion difference was not statistically significant, with a p value of 0.40. These results indicate that the two groups were similar with respect to age and gender, while BMI was significantly higher among carriers.

Table 2:Quantitative Comparison of Bacterial and Toxin Loads Between Carrier and Control Groups

Variable	Carrier Group (n = 25)	Control Group (n = 25)	p value
Total Bacterial Load 16S (copies/g)	102.8×10^9 ($\pm 18.2 \times 10^9$)	97.7×10^9 ($\pm 19.3 \times 10^9$)	0.38
tcdA Load (copies/g)	11.0×10^6 ($\pm 2.3 \times 10^6$)	598 (±269)	<0.0001
tcdB Load (copies/g)	9.8×10^6 ($\pm 2.9 \times 10^6$)	419 (±310)	<0.0001

Results are presented as mean ± standard deviation. Independent samples t-test was used for all comparisons under the assumption of approximate normality. A p value less than 0.05 was considered statistically significant.

The comparative statistical analysis of quantitative microbial data between the two groups revealed marked differences in the presence of *Clostridioides difficile* toxin genes. Although the mean total bacterial load measured by 16S rRNA gene copies per gram of stool was slightly higher in the carrier group (mean of 102.8×10^9 copies/g) compared to the control group (97.7×10^9 copies/g), this difference was not statistically significant with a p value of 0.38, indicating that the overall microbial burden in fecal samples was comparable between groups.

In contrast, the toxin gene loads showed dramatic and statistically significant elevations in the carrier group. The mean load of the *tcdA* gene was 11.0 million copies per gram with a standard deviation of 2.3 million in carriers, while in the control group, the mean *tcdA* load was extremely low at only 598 copies per gram. This stark contrast was highly significant with a p value below 0.0001. Similarly, the mean *tcdB* gene load in the carrier group reached 9.8 million copies per gram with a standard deviation of 2.9 million, whereas in controls, the levels were virtually negligible, with an average of 419 copies per gram. This difference was again statistically significant, with a p value less than 0.0001.

Taken together, the data show that while total bacterial loads did not differ significantly between groups, the presence and magnitude of expression of *C. difficile* toxin genes were substantially and significantly elevated in carriers relative to controls. This strongly supports the biological distinction in pathogenic potential between the two groups, reflective of the carrier status defined by the presence of *tcdA* and *tcdB*.

Table 3:Comparative Analysis of Fecal Short-Chain Fatty Acid Concentrations Between Carrier and Control Groups

SCFA (mmol/kg)	Carrier Group (n = 25)	Control Group (n = 25)	p value
Acetate	50.58 ± 9.56	60.64 ± 8.18	<0.0001
Propionate	17.79 ± 4.34	22.16 ± 3.69	<0.0001
Butyrate	11.13 ± 2.00	16.10 ± 4.21	<0.0001

Data are presented as mean ± standard deviation. Two-tailed independent samples t-test was used to compare means between groups. A p value less than 0.05 was considered statistically significant.

The statistical analysis of short-chain fatty acids (SCFAs) in stool samples revealed significant differences between individuals in the carrier group and those in the control group. For acetate, the mean concentration in carriers was 50.58 mmol/kg with a standard deviation of 9.56, compared to a significantly higher mean of 60.64 mmol/kg in controls with a standard deviation of 8.18. This difference was statistically significant, with a p value of less than 0.0001, suggesting a notable reduction in acetate among carriers.

Propionate levels also followed a similar pattern. The carrier group showed a mean concentration of 17.79 mmol/kg with a standard deviation of 4.34, while the control group had a higher mean of 22.16 mmol/kg and a standard deviation of 3.69. The p value for this comparison also fell below 0.0001, confirming the statistical significance of the difference and indicating a consistent reduction in this SCFA among carriers.

The most pronounced contrast was observed in butyrate concentrations. Carriers exhibited a mean level of 11.13 mmol/kg with a standard deviation of 2.00, whereas controls had an average concentration of 16.10 mmol/kg with a standard deviation of 4.21. The difference between groups was again highly significant, with a p value under 0.0001. This suggests a substantial reduction in butyrate production or availability in carriers when compared to controls.

Taken together, the data strongly indicate that colonization is associated with reduced levels of key gut SCFAs—acetate, propionate, and butyrate—all of which were significantly lower in carriers than in healthy controls. This may reflect alterations in gut microbiota composition or metabolic activity associated with the carrier state.

Table 4: Comparison of Fecal Calprotectin Levels Between Carrier and Control Groups

Variable	Carrier Group (n = 25)	Control Group (n = 25)	p value
Fecal Calprotectin (mg/kg)	102.8 ± 65.2	44.3 ± 23.8	<0.0001

Values are expressed as mean ± standard deviation. Normality was assessed using the Shapiro–Wilk test. Independent samples t-test was applied under assumption of normal distribution. A p value less than 0.05 was considered statistically significant.

The comparative analysis of fecal calprotectin concentrations between the two study groups revealed a statistically significant elevation in the carrier group relative to controls. The average fecal calprotectin concentration of carriers was 102.8 mg/kg with a significantly broad standard deviation of 65.2, as there was a lot of variability in the population, probably because of the varying levels of subclinical inflammation or immune response. On the contrary, the control group had a significantly lower mean of 44.3mg/kg with a standard deviation of 23.8. The result of this statistic was very significant and the p value was below 0.0001 and this means that the difference in the levels of calprotectin between the two groups cannot be attributed to chance.

This pronounced increase of fecal calprotectin in carriers indicates a higher level of inflammatory condition in the intestinal mucosa, which can be caused by the microbial colonization or by the reaction of the host against the bacterial toxins. Carrier values were highly variable with some having very low values and some having abnormally high concentrations indicating heterogeneity of carrier state. On the other hand, the control group had a more limited distribution of values with a range in the lower side, which is normal in healthy people without any visible gastrointestinal inflammation.

Collectively, the findings indicate that clinically silent asymptomatic carriers of pathogenic organisms may possess quantifiable mucosal inflammation as indicated by their significantly high levels of calprotectin. This observation highlights the possible subclinical effects of colonization and gives credence to the applicability of inflammation-sensitive biomarkers when differentiating microbiologically diverse patient populations.

Table 5: Logistic Regression Analysis: Predictors of Asymptomatic C. difficile Carriage

Variable	Coefficient	Odds Ratio	95% CI Lower	95% CI Upper	p value
BMI	0.5946	1.812	1.035	3.172	0.0374
Butyrate (mmol/kg)	-0.5487	0.578	0.365	0.913	0.0189
Fecal Calprotectin (mg/kg)	0.0693	1.072	1.014	1.133	0.0150
Acetate (mmol/kg)	-0.1456	0.865	0.752	0.994	0.0416
Age	-0.0521	0.949	0.834	1.081	0.4313

Logistic regression analysis was used to identify significant predictors of asymptomatic C. difficile carriage, with the dependent variable set as group assignment (Carrier vs. Control). Independent variables included BMI, Butyrate (mmol/kg), Fecal Calprotectin (mg/kg), Acetate (mmol/kg), and Age. Reported statistics include regression coefficients, odds ratios (OR), 95% confidence interval bounds for the OR, and p values. A p value less than 0.05 was considered statistically significant.

Among the variables examined as potential predictors for asymptomatic carriage, BMI demonstrated a significant positive association. For each unit increase in BMI, the odds of being a carrier increased by 81.2%, as expressed by an OR of 1.812 (p = 0.0374). This suggests that higher BMI is independently associated with increased risk of carriage.

Butyrate concentration in stool exhibited a significant inverse association: as butyrate levels increased, the odds of carriage decreased. The odds ratio for butyrate was 0.578, with a 95% confidence interval excluding unity (p = 0.0189), indicating that higher butyrate concentrations are protective against carriage.

Fecal calprotectin was also a significant predictor, with an odds ratio of 1.072 per mg/kg increase (p = 0.0150). This supports the interpretation that increasing gut inflammation, reflected by higher calprotectin levels, is independently associated with greater odds of being an asymptomatic carrier.

Acetate concentration emerged as an additional protective factor. Each mmol/kg increment in fecal acetate resulted in an approximately 13.5% reduction in odds of carriage, with an OR of 0.865 and a significant p value of 0.0416, supporting the negative relationship between this short-chain fatty acid and carrier status.

Age did not show a significant effect, with an odds ratio of 0.949 (p = 0.4313), and the confidence interval included unity. This suggests that, when accounting for the other factors in the model, age alone is not an independent predictor of asymptomatic carriage in this cohort.

In conclusion, logistic regression analysis identifies higher BMI and calprotectin as independent risk factors for carriage, while greater levels of butyrate and acetate serve as protective factors. These findings highlight the relevance of metabolic and inflammatory markers in the gut environment as determinants of asymptomatic C. difficile colonization.

Table 6; Multivariate Linear Regression Analysis: Determinants of Fecal Calprotectin Levels

Variable	Coefficient	Std. Error	t-value	p value	95% CI Lower	95% CI Upper
Intercept	96.47	79.89	1.21	0.234	-64.54	257.48
tcdA Load (copies/g)	6.07E-06	2.56E-06	2.37	0.022	9.02E-07	1.12E-05
tcdB Load (copies/g)	-6.19E-07	2.62E-06	-0.24	0.814	-5.90E-06	4.66E-06
Butyrate (mmol/kg)	0.35	2.15	0.16	0.871	-3.98	4.68
BMI	-2.58	1.95	-1.33	0.192	-6.51	1.34
Total Bacterial Load 16S (copies/g)	1.22E-10	3.62E-10	0.34	0.738	-6.08E-10	8.52E-10

Multivariate linear regression was performed with fecal calprotectin (mg/kg) as the dependent variable, and tcdA Load (copies/g), tcdB Load (copies/g), Butyrate (mmol/kg), BMI, and Total Bacterial Load (copies/g) as independent variables. A p value less than 0.05 was considered statistically significant.

The multivariate linear regression analysis showed that of the chosen predictors, only tcdA Load had a statistically significant correlation with fecal calprotectin values, with a coefficient of 6.07 x 10 -6 and a p value of 0.022. This implies that even after correcting all other variables in the model, the burden of C. difficile tcdA is independently linked to elevated fecal calprotectin concentrations suggesting a direct association between burden of C. difficile tcdA and a marker of intestinal inflammation.

The other predictors in the model such as tcdB Load, butyrate levels, BMI, and total bacterial load were not found to be statistically significant in association with fecal calprotectin. The tcdB Load coefficient was not significant and negative, so there was no independent linear association between tcdB Load and fecal calprotectin in the current analysis. Butyrate and total bacterial load also had non-significant coefficients with wide confidence intervals crossing zero, implying a lack of evidence for direct effects on calprotectin levels after adjusting for the presence of the other variables. BMI, although showing a negative association with calprotectin, did not reach significance, suggesting that body mass index does not independently predict intestinal inflammation as measured by calprotectin within this study population when considering all covariates together.

The confidence intervals for most variables are wide, reflecting substantial variability and uncertainty in the estimates for these predictors, apart from tcdA Load, where both the coefficient and its confidence bounds exclude zero, confirming its significant positive association. Collectively, these findings emphasize that, when considering multiple relevant factors simultaneously, tcdA Load emerges as the primary independent determinant of fecal calprotectin in this cohort, reinforcing the link between toxigenic C. difficile carriage and subclinical gut inflammation. Other microbial and host factors, including total butyrate production, total microbial burden, and BMI, do not appear to exert a significant independent effect on inflammation biomarker levels within this multivariate framework.

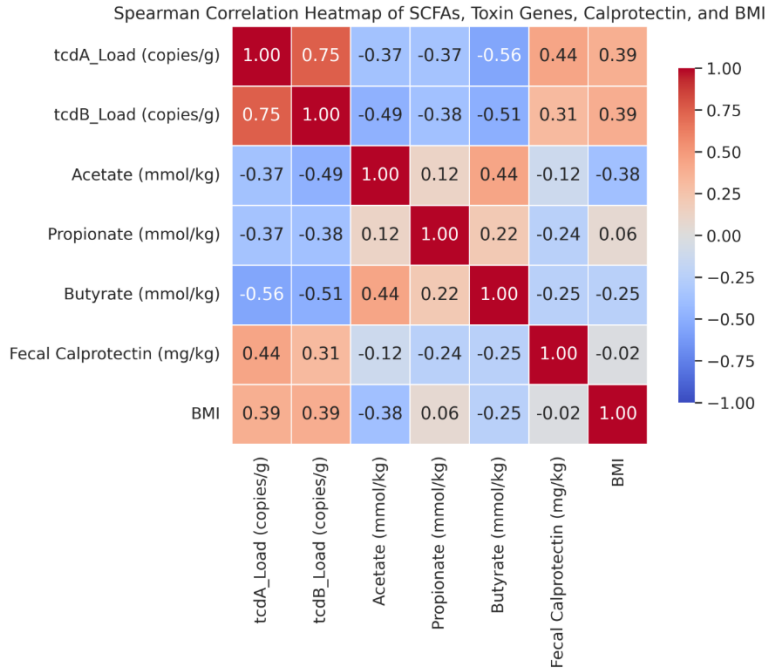

Figure 1. Spearman Correlation Heatmap of Toxin Gene Loads, SCFA Concentrations, Calprotectin, and BMI

This heatmap illustrates the pairwise Spearman correlation coefficients among key microbial, metabolic, and host inflammatory variables. Notably, butyrate levels showed inverse correlations with both *tcdA* and *tcdB* gene loads, as well as fecal calprotectin, suggesting a protective metabolic signature against inflammation and toxigenic colonization. Acetate and propionate exhibited moderate negative associations with inflammatory markers. Fecal calprotectin was positively correlated with *tcdA*, reinforcing its role as a marker of subclinical mucosal inflammation. The heatmap captures complex interactions and highlights the metabolic–inflammatory axis underlying asymptomatic *C. difficile* colonization.

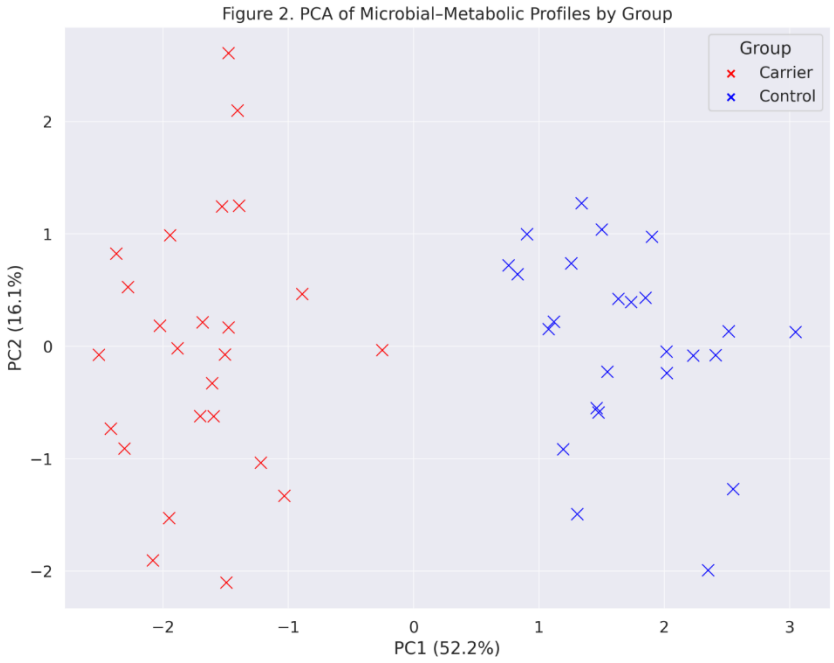

Figure 2. Principal Component Analysis (PCA) of Microbial–Metabolic Profiles in Asymptomatic Carriers vs. Controls

This PCA plot visualizes the distribution of participants based on integrated microbial and metabolic variables, including SCFAs (acetate, propionate, butyrate), toxin gene loads (*tcdA*, *tcdB*), and fecal calprotectin levels. Each point represents an individual, color-coded by group. The separation along the principal components indicates distinct clustering between asymptomatic *C. difficile* carriers and healthy controls. PC1 and PC2 collectively capture the majority of variance in the data. The observed divergence highlights the contribution of combined microbial and host factors in defining the carrier state and supports the biological differentiation between groups at the metabolic-inflammation interface.

3. DISCUSSION

The present study demonstrates a statistically significant elevation in both tcdA and tcdB gene loads among asymptomatic Clostridioides difficile carriers when compared to healthy controls, while total bacterial load remained statistically indistinct between the groups. This suggests that the asymptomatic carrier state is not defined by a global overgrowth of bacteria but by the selective enrichment of toxigenic C. difficile strains. Clinically, this finding raises important questions about the potential pathogenic readiness of carriers, who despite lacking symptoms, may harbor high levels of virulence genes and thus pose a risk of transmission or progression to symptomatic disease, especially under conditions like antibiotic use or immune suppression.

A longitudinal study of pediatric acute lymphoblastic leukemia (ALL) patients suggests complementary support of the current results, indicating an increased prevalence of asymptomatic toxigenic C. difficile carriage during treatment, especially in cases of higher tcdA, tcdB gene detections using qPCR. These data confirm the opinion that carriage is possible even at high toxigenic gene loads without obvious symptoms, particularly in susceptible groups with dysbiosis (Yang et al., 2021).

Similar to the present work, Kordus et al. (2023) employed innovative nanobody-driven quantitative measures to demonstrate that the expression of TcdA and TcdB is highly variable in vivo, with the expression of toxin genes usually preceding toxin-induced pathology. This means that the presence of toxin genes might not be an adequate measure of active infection but can still reflect a colonization state with a potential latent pathogenic ability (17).

Similar results are provided by Kim et al. (2018), who found that the tcdB gene loads, measured as Ct values, were significantly associated with the presence of toxins, though not with the clinical disease severity. This again supports the notion that the presence of high toxin gene loads as described in the current study may not directly translate to clinical symptoms but rather act as surrogates of transmission potentiality and colonization propensity (18).

Unlike the focus of the current study on an increased load of toxin genes in asymptomatic carriers, Takemori-Sakai et al. (2022) isolated C. difficile strains that did not have a full tcdA gene yet were positive in toxin detection tests. This implies that a strain may have divergent gene presence and functionality, and that genomic deletions or mutations that can reduce toxin functionality may be present, even though a gene is present (19).

There is additional contention with Gao et al. (2020) who demonstrated that although tcdB expression causes substantial intracellular effects on the gene expression of epithelial cells, the mere presence of the gene did not imply pathological consequences unless it was expressed to a functional level. Therefore, the current study demonstrates the presence of the genes but does not indicate active toxin expression and functional cytotoxicity in vivo, particularly in asymptomatic infections (20).

A different contradictory result is presented by Carter et al. (2015) who gave evidence in animal models that TcdB alone is capable of causing both localized and systemic disease and that the presence of TcdB alone strongly correlates with pathogenicity. This refutes the observation of the current study that such large gene loads may be in a clinically silent state in that the pathological manifestation of these toxins in asymptomatic carriers is probably being suppressed by host factors or immune modulation (21).

The difference between the studies can be justified by a number of reasons. First, the host immune response and the composition of the gut microbiota are likely to modulate the effect of the presence of toxin genes, allowing high loads in certain individuals without causing symptomatic infection. Second, variation in strain, such as mutations that alter the expression or secretion of toxins, may be the cause of variation. Lastly, methodological variation- including the presence of gene, mRNA expression, or active toxin protein- also play a role in the difference in interpretation of a clinically significant toxigenic burden.

In combination, although the present research proves the high level of tcdA and tcdB genes in asymptomatic people, the comparison with recent publications indicates some consistency and inconsistencies. This highlights the complicated interrelationship between microbial factors, host defense, and methodological instruments in outlining the meaning of toxigenic gene carriage. More subtle diagnostics that combine gene, transcript, and protein level assessments may be needed to properly stratify risk to patients and direct infection control.

The current research showed that there were significantly lower concentrations of acetate, propionate, and butyrate in the stool of asymptomatic Clostridioides difficile carriers than in healthy controls. Such results indicate an aberrant metabolic

environment in carriers, which may be indicative of gut dysbiosis or microbial composition change. Such decreases in short-chain fatty acids (SCFAs) clinically can negatively affect mucosal immunity, epithelial barrier integrity, and colonization resistance, which are highly dependent on SCFA metabolism. Butyrate, specifically, has been described as anti-inflammatory and epithelial protective, and its loss is of particular concern when it comes to gut homeostasis maintenance.

These results are well correlated with a number of recent works. In particular, Sayol-Altarriba et al. (2024) found that the levels of butyrate were always lower in dysbiotic stool samples of CDI patients, and when adjusted to the number of bacteria, butyrate had the highest discriminative ability between eubiotic and dysbiotic microbiomes. This can be compared with the findings of the present study and validate the position of butyrate as an indicator of gut health and resistance to C. difficile colonization (22).

In the same manner, Jess et al. (2023) showed that the levels of acetate, propionate, and butyrate in pediatric patients with recurrent CDI were significantly lower than in healthy controls. Such levels increased after fecal microbiota transplantation (FMT), which further supports the idea that SCFA loss is associated with CDI susceptibility and that replenishment can have a therapeutic effect (23).

Arcay et al. (2024) also observed a decrease in the amount of SCFA-producing bacteria in patients with CDI, in particular, microbial richness decreased, and the number of bile salt hydrolase-producing organisms increased. This metabolic alteration was coupled with a loss of advantageous lipids and fatty acids, which implies a more comprehensive metabolic failure related to CDI, as depicted by the SCFA loss in the current study (24).

According to Gregory et al. (2021), SCFAs also play a role in regulating C. difficile virulence and host immune responses. In their review, they suggested a model whereby the C. difficile perceives low SCFA as an indicator of dysbiosis and adjusts to it by increasing its virulence, which further distorts the balance of the gut. The mechanistic understanding provides a conceptual framework to the findings of the present study that low SCFAs in carriers are not necessarily harmless but would allow toxin genes expression and inflammation (25).

Moreover, Ouyang et al. (2022) highlighted SCFAs' therapeutic potential, noting that they suppress inflammation and inhibit C. difficile growth in vitro and in vivo. The review supports a protective role for SCFAs and points to their depletion as a key vulnerability in host defenses against CDI, again aligning with the present study's implications (26).

However, not all findings are congruent with the present study. Jo et al. (2025) in a controlled trial found that vancomycin treatment in healthy volunteers significantly decreased SCFA levels, but also observed that SCFA depletion did not uniformly translate to increased C. difficile pathogenicity in all participants. This suggests that SCFA levels alone may not fully explain susceptibility, especially in the absence of other dysbiotic triggers (27).

Contrasting evidence also comes from Hayashi and Kamada (2020), who reported that administration of butyrate-producing Clostridium butyricum improved survival in a murine CDI model, but the effect was mediated independently of SCFA receptors GPR43 and GPR109a. This suggests that while SCFA levels matter, their physiological impact may vary depending on host receptor functionality, which was not assessed in the present study (28).

Taken together, the current study provides strong evidence that reduced levels of fecal SCFAs, particularly butyrate and acetate, are associated with asymptomatic C. difficile carriage. This aligns with a growing body of literature linking SCFA depletion to dysbiosis and CDI susceptibility. However, some studies highlight the complexity of host-microbe interactions and indicate that SCFA depletion alone may not fully predict disease progression. Variability in host response pathways, microbial context, and environmental factors may modulate the clinical impact of reduced SCFAs. Therefore, while SCFA profiling is a promising non-invasive biomarker, its predictive value will be enhanced when integrated with other microbiome and immune parameters.

The present study reported a significantly elevated mean fecal calprotectin (FC) level in asymptomatic Clostridioides difficile carriers (102.8 mg/kg) compared to healthy controls (44.3 mg/kg), with a highly significant p value (<0.0001). These findings suggest that, despite the absence of overt clinical symptoms, asymptomatic colonization is associated with a state of subclinical mucosal inflammation. The wide variability in FC levels among carriers indicates heterogeneous immune responses to microbial colonization and possible early inflammatory signaling. These results propose that FC could be used as a non-invasive biomarker for detecting hidden inflammatory activity and risk stratification in C. difficile-colonized individuals.

Supporting the current study's findings, Han et al. (2020) found significantly elevated fecal calprotectin in patients colonized with toxigenic C. difficile compared to non-colonized controls, although the levels were even higher in those with active C. difficile infection (CDI). This implies that colonization alone can incite measurable inflammation, consistent with the idea that asymptomatic carriers are not entirely immunologically silent (29).

Similarly, Suárez-Carantoña et al. (2021) observed that FC levels were low in patients with presumed colonization or mild CDI, but elevated in those with active, clinically diagnosed CDI, suggesting that FC levels can vary across the clinical spectrum of C. difficile interactions. However, their lowest FC group still showed non-zero calprotectin levels, supporting

the current study's observation of low-grade inflammation in colonized but asymptomatic subjects (30).

Additional validation comes from Doppalapudi et al. (2025), who demonstrated that elevated FC was associated with both primary and recurrent CDI, and that higher levels correlated with more severe disease and recurrence. Although their focus was on active infection, the paper noted that asymptomatic carriers can exhibit elevated FC as a predictor of poor outcomes, aligning with the subclinical implications raised in the present study (31).

Contrasting the current findings, Wen et al. (2022) conducted a systematic review and concluded that while FC might reflect disease severity in symptomatic CDI, its utility as a diagnostic or prognostic tool remains limited due to variability in levels and lack of standardization. They did not find strong evidence to support FC as a reliable marker in asymptomatic patients, thereby questioning its predictive power in non-clinical settings (32).

Further dissent is found in the review by Yusuf et al. (2020), who reported inconsistent associations between FC levels and CDI severity, with some studies showing significance and others not. The lack of agreement across methodologies and definitions of severity undermined FC's reliability in various CDI contexts, including colonization (33).

Moreover, Fernández-Ruiz et al. (2023) found that although elevated FC was linked to recurrence and severity in CDI, it lacked adequate specificity to serve as a standalone predictor. Their findings underline the challenge of interpreting intermediate FC levels and caution against over-reliance on FC in ambiguous clinical presentations like asymptomatic colonization (34).

Overall, the present study's finding of elevated FC in asymptomatic carriers is in agreement with several studies indicating a relationship between colonization and subclinical inflammation. However, other studies question the diagnostic specificity and reproducibility of FC measurements across clinical settings. This discrepancy likely arises from variations in study design, cut-off values, population characteristics, and assay methodologies. Despite these limitations, the consistent signal of elevated FC in carriers found in the current study supports its potential role as a surveillance biomarker, although its standalone diagnostic utility remains debated.

4. CONCLUSION

This study demonstrates that asymptomatic *C. difficile* carriers exhibit significantly elevated fecal toxin gene loads, reduced concentrations of key SCFAs, and increased fecal calprotectin levels—indicative of a metabolically and immunologically distinct state from healthy controls. The data support a model in which SCFA depletion and subclinical inflammation co-occur with toxigenic colonization, possibly predisposing to symptomatic progression. Logistic regression identified higher BMI and calprotectin as risk factors for colonization, while higher butyrate and acetate were protective. Multivariate analysis implicated *tcdA* gene load as a key driver of calprotectin elevation. These findings underscore the utility of integrated molecular-metabolic profiling in identifying high-risk asymptomatic carriers and improving CDI risk stratification

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