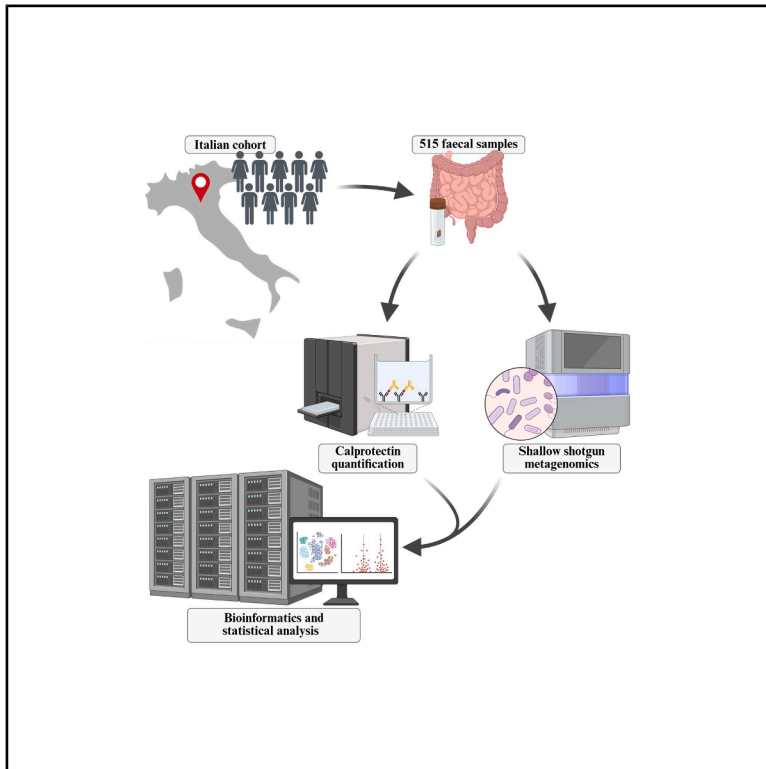


# Fecal calprotectin and gut microbiome in a cohort without intestinal pathologies from northern Italy

## Graphical abstract



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## In brief

Gastroenterology; Precision medicine;  
Microbial genomics; Microbiome;  
Systems biology

## Highlights

- Butyrate-producing bacteria are negatively correlated with calprotectin
- Opportunistic pathogens are positively correlated with calprotectin
- Functional microbial metabolic signatures correlated with calprotectin levels
- Microbiota features may provide early pre-clinical biomarkers of gut inflammation



## Article

# Fecal calprotectin and gut microbiome in a cohort without intestinal pathologies from northern Italy

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## SUMMARY

Fecal calprotectin is a biomarker for intestinal inflammatory conditions, while specific taxa of gut microbiota are proposed as biomarkers for inflammatory bowel disease. However, the relationship between microbiota and calprotectin levels is still largely unexplored. Using shallow shotgun metagenomics, we investigate microbial taxonomic and functional patterns correlated with calprotectin levels in fecal samples of 515 adult individuals without known intestinal pathologies, enrolled within the Parma Microbiota Project. The median value of calprotectin was 23.6  $\mu\text{g/g}$ , but levels higher than the normal threshold of 50  $\mu\text{g/g}$  were measured in 20% of participants. While no changes were detected in alpha- and beta-diversities, calprotectin levels were negatively associated with butyrate-producing bacteria, while positively correlated with several opportunistic pathogens. Functional analysis showed significant correlations between calprotectin levels and the predicted microbial enzymatic functions. If confirmed in longitudinal studies, these findings could indicate early microbial biomarkers of gut inflammatory conditions.

## INTRODUCTION

Inflammatory bowel disease (IBD) is a heterogeneous group of multifactorial chronic and relapsing inflammatory pathologies of the gastrointestinal tract, with Crohn's disease (CD) and ulcerative colitis (UC) representing the two extreme phenotypes.<sup>1</sup> IBD is characterized by a complex pathogenesis involving genetic predisposition, environmental factors, and gut microbiota composition.<sup>2</sup> The gold standard to establish IBD diagnosis is endoscopy, which is a costly and invasive procedure. Therefore, non-invasive, specific, and sensitive biomarkers are badly needed to suggest early diagnosis and to assess disease activity, therapy response, and recurrence.<sup>3</sup>

The main IBD biomarker is fecal calprotectin (FC), a calcium-binding protein belonging to the S100 family composed of the monomers S100A8 and S100A9. Calprotectin is released by neutrophils in the intestinal lumen, reflecting the local inflammatory status.<sup>4</sup> In the extracellular environment, FC chelates divalent metal ions (e.g., calcium, iron, magnesium, and zinc), thus limiting the growth of invasive and commensal gut bacteria.

This binding causes a conformational change of FC, which becomes resistant to the proteolytic intestinal environment, thus allowing its determination in stool samples. FC is a sensitive IBD biomarker and can reach very high levels in patients (CD,  $\sim 3,200 \mu\text{g/g}$ ; UC,  $\sim 1,900 \mu\text{g/g}$ ),<sup>5</sup> but it is not highly specific since several confounding factors may alter its levels.<sup>6</sup>

In this scenario, specific bacteria have been proposed as potential disease biomarkers.<sup>7–10</sup> Alterations in the microbiota composition from a state of equilibrium (i.e., eubiosis) to an unbalanced state (i.e., dysbiosis) have been reported in IBD patients.<sup>11</sup> Therefore, disentangling the gut microbiota composition could represent a non-invasive tool to complement FC determination in evaluating the development or recurrence of IBD.<sup>12</sup> However, the relationship between FC and microbiota has not been extensively investigated.<sup>13</sup>

The Parma Microbiota Project,<sup>14</sup> an ongoing study at the University of Parma, aims to profile the fecal microbiota of an adult population from a specific area of northern Italy without known intestinal pathologies and to identify microbial features potentially associated with inflammatory conditions. We have



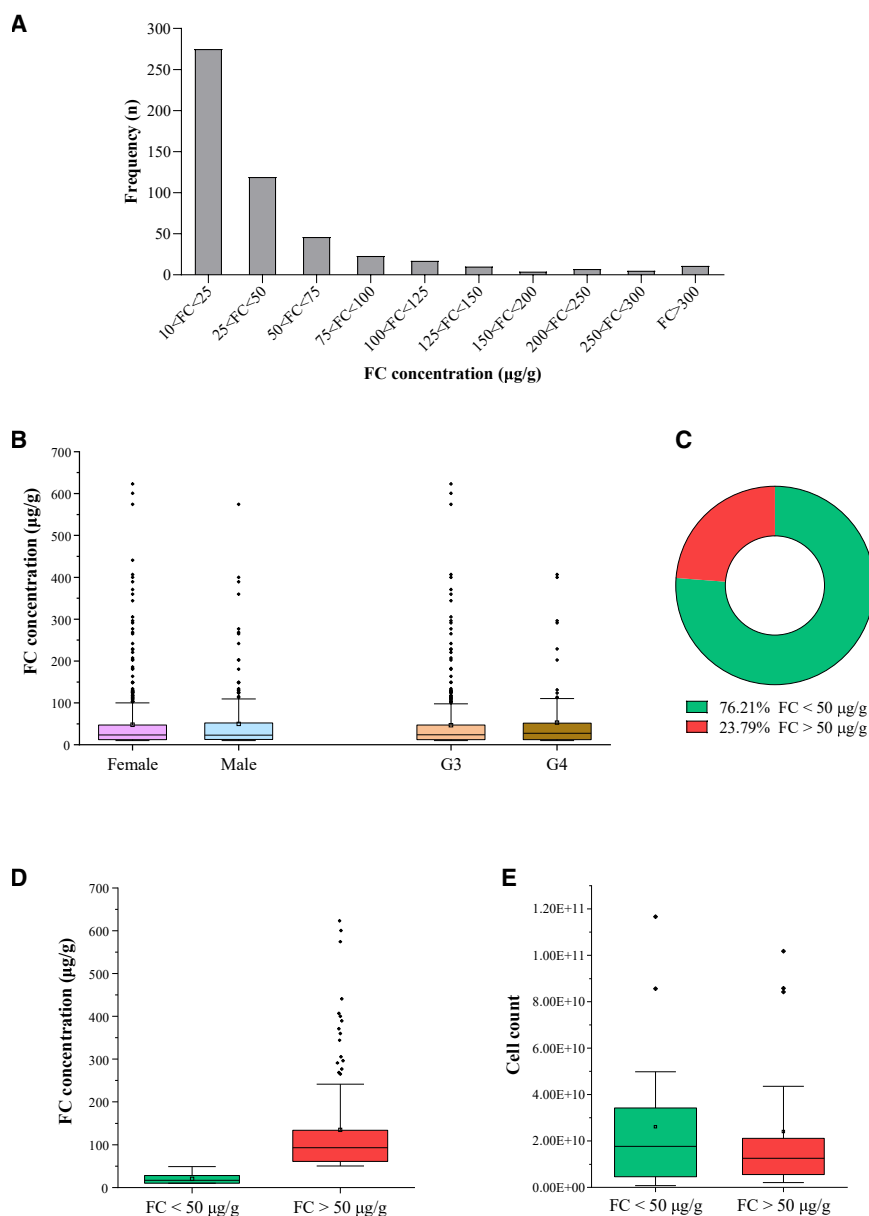


**Figure 1. FC levels in adult population from Parma Microbiota cohort**

(A) Distribution of FC levels across different concentration intervals in the study cohort ( $n = 515$ ). (B) Correlation between FC levels, age, and gender (Mann-Whitney U test  $p$  values  $>0.05$ ). Boxes represent the interquartile range (IQR), 25th–75th percentiles), whiskers extend to 1.5 times the IQR, and the line within the boxes indicates the median. (C) Distribution of FC levels into two groups: normal (FC  $< 50$  µg/g) and high (FC  $> 50$  µg/g). (D) FC concentration average of normal and high groups. See (B) for the meaning of boxes and whiskers. (E) Comparison of total bacterial load, measured by flow cytometry, between individuals with normal FC ( $n = 20$ ) and high FC ( $n = 20$ ). Bacterial counts are expressed as cells/mL of fecal suspension. See (B) for the meaning of boxes and whiskers.

over 50% of individuals with FC levels below 25 µg/g ( $n = 275$ ) and with the number of individuals in the subsequent ranges decreasing as the FC value increases (Figure 1A).

The samples were divided into two age groups, G3 (18–64 years) and G4 (65 years and older), following the guidelines provided by the World Health Organization (WHO),<sup>15</sup> and a possible correlation between the FC levels, sex, and the two age groups was evaluated. Neither gender nor age influenced the FC levels in adults (Mann-Whitney U test  $p$  values  $>0.05$ ) (Figure 1B).

Subsequently, data were divided into two groups corresponding to different FC levels, normal (FC  $< 50$  µg/g) and high (FC  $> 50$  µg/g), considering normal FC levels in a healthy adult population between 10 and 50 µg/g.<sup>4</sup> More than 70% of individuals showed FC levels below the

collected a wide range of health-related parameters from participants, to consider potential confounding factors, and evaluated taxonomic and functional microbiome profiles to investigate potential links between microbiota composition, microbial metabolic pathways, and FC levels.

## RESULTS

### Calprotectin levels in fecal samples from Parma Microbiota cohort

The mean FC of the enrolled 515 adult individuals (60% women, 40% men; mean age  $50.4 \pm 15.1$  [SD] years) was  $47.4$  µg/g  $\pm$   $74.67$  (SD), while the median was  $23.6$  µg/g (Q1  $12.1$ –Q3  $47.1$ ). The frequency of the FC values showed a hyperbolic trend, with the highest point of the curve represented by

cutoff considered normal (50 µg/g), with a mean value of  $20.3$  µg/g  $\pm$   $10.73$  (SD) and a median of  $17.1$  µg/g (Q1  $10.0$ –Q3  $28.8$ ). The remaining part showed mean FC levels of  $134.2$  µg/g  $\pm$   $115.12$  (SD) and a median of  $93.2$  µg/g (Q1  $61.0$ –Q3  $132.4$ ), indicating higher heterogeneity and a broader distribution of FC values within this subgroup, which may be consistent with an altered intestinal status (Figures 1C and 1D).

To investigate the potential impact of FC levels on bacterial load, a subset of 40 samples, i.e., 20 with normal and 20 with high FC, was analyzed using flow cytometry to enumerate the bacterial cells. The results revealed high interindividual variability and no significant difference between the groups (Mann-Whitney U test  $p$  value  $>0.05$ ), with an average bacterial load of  $2.61 \pm 3.01 \times 10^{10}$  cells/mL in the normal group and  $2.41 \pm 3.00 \times 10^{10}$  cells/mL in the high group (Figure 1E).

**Table 1. Descriptive statistics of potential factors influencing fecal calprotectin levels**

|                                                               | FC normal (<50 µg) |                 |       | FC high (>50 µg) |                 |       | p value      |
|---------------------------------------------------------------|--------------------|-----------------|-------|------------------|-----------------|-------|--------------|
|                                                               | Number/mean        | Number metadata | %     | Number/mean      | Number metadata | %     |              |
| Female (n)                                                    | 239                | 388             | 61.60 | 68               | 123             | 55.28 | >0.05        |
| Male (n)                                                      | 149                | 388             | 38.40 | 55               | 123             | 44.72 | >0.05        |
| Age (mean ± SD)                                               | 50.3 ± 14.8        | 354             | –     | 50.7 ± 16.1      | 111             | –     | >0.05        |
| BMI (mean ± SD)                                               | 23.5 ± 3.7         | 294             | –     | 24.0 ± 4.5       | 90              | –     | >0.05        |
| Diabetes (n)                                                  | 8                  | 289             | 2.77  | 7                | 88              | 7.95  | >0.05        |
| Metabolic syndrome (n)                                        | 9                  | 282             | 3.19  | 2                | 85              | 2.35  | >0.05        |
| Dyslipidemia (n)                                              | 33                 | 280             | 11.79 | 8                | 87              | 9.20  | >0.05        |
| Arterial hypertension (n)                                     | 53                 | 290             | 18.28 | 21               | 87              | 24.14 | >0.05        |
| Ischemic cardiomyopathy (n)                                   | 4                  | 283             | 1.41  | 0                | 88              | 0.00  | >0.05        |
| Peripheral vascular disease (n)                               | 8                  | 284             | 2.82  | 2                | 88              | 2.27  | >0.05        |
| Thyroid pathology (n)                                         | 56                 | 284             | 19.72 | 24               | 88              | 27.27 | >0.05        |
| Altered bowel habit (n)                                       | 36                 | 267             | 13.48 | 14               | 83              | 16.87 | >0.05        |
| Past SARS-CoV-2 (n)                                           | 122                | 259             | 47.10 | 38               | 81              | 46.91 | >0.05        |
| Recurrent infections (n)                                      | 4                  | 281             | 1.42  | 3                | 85              | 3.53  | >0.05        |
| Depressive disorder (n)                                       | 16                 | 281             | 5.69  | 8                | 88              | 9.09  | >0.05        |
| Anxiety disorders (n)                                         | 34                 | 284             | 11.97 | 15               | 86              | 17.44 | >0.05        |
| Alcohol consumption (n)                                       | –                  | –               | –     | –                | –               | –     | <b>0.029</b> |
| Yes                                                           | 229                | 294             | 77.89 | 66               | 90              | 73.33 | –            |
| No                                                            | 64                 | 294             | 21.77 | 20               | 90              | 22.22 | –            |
| Ex                                                            | 1                  | 294             | 0.34  | 4                | 90              | 4.44  | –            |
| Smokers (n)                                                   | –                  | –               | –     | –                | –               | –     | >0.05        |
| Yes                                                           | 41                 | 290             | 14.14 | 7                | 88              | 7.95  | –            |
| No                                                            | 168                | 290             | 57.93 | 56               | 88              | 63.64 | –            |
| Ex                                                            | 81                 | 290             | 27.93 | 25               | 88              | 28.41 | –            |
| Chronic PPI use (n)                                           | 21                 | 252             | 8.33  | 14               | 73              | 19.18 | <b>0.016</b> |
| Chronic paracetamol use (n)                                   | 37                 | 263             | 14.07 | 13               | 76              | 17.11 | >0.05        |
| Chronic NSAIDs (Non-Steroidal Anti-Inflammatory Drugs use (n) | 21                 | 262             | 8.02  | 6                | 75              | 8.00  | >0.05        |
| Chronic corticosteroids use (n)                               | 7                  | 263             | 2.66  | 4                | 73              | 5.48  | >0.05        |
| Iron integration (n)                                          | 8                  | 265             | 3.02  | 2                | 76              | 2.63  | >0.05        |
| Vitamins integration (n)                                      | 87                 | 267             | 32.58 | 21               | 78              | 26.92 | >0.05        |
| Other integrations (n)                                        | 95                 | 267             | 35.58 | 19               | 78              | 24.36 | >0.05        |

Based on the available completed questionnaires, the influence of potential confounding factors on FC levels was evaluated. These included body mass index (BMI), chronic diseases, anxiety/depressive disorders, infections, intestinal habits, smoking/alcohol consumption, drug use, and dietary supplement intake. In most cases, no significant differences were obtained, except for alcohol consumption and, more evidently, for chronic use of proton pump inhibitors (PPIs), which was more frequent in the group with FC levels >50 µg/g (Table 1).

### Correlation between microbiota biodiversity and calprotectin levels

For the metagenomic analysis, the 515 stool samples were utilized to determine microbiota composition using the METAnnotatorX2 software,<sup>16,17</sup> adhering to the standard filtering parameters outlined in the manual, including the removal of *Homo*

*sapiens* reads. The downloaded fastq files were processed using the same bioinformatics pipeline to prevent biases. This process yielded a total of 3,717,226,696 reads with an average per sample of  $7,217,916 \pm 2,633,349$  after quality and human sequence filtering (Table S1). Taxonomic profiling was intentionally performed according to a shallow shotgun metagenomic approach,<sup>16,18</sup> with a random subset of up to 100,000 reads per sample analyzed after quality control and human sequence filtering,<sup>19</sup> obtaining a total of 29,632,512 classified reads with an average of  $57,539 \pm 7,986$  reads per sample (Table S1). As previously reported, this method optimized the bioinformatics pipeline, ensuring precise taxonomic profiling and enabling reliable comparisons by reducing the impact of variations in the total number of analyzed reads.<sup>16,18</sup> All downstream taxonomic analyses, including biodiversity analyses and taxon association testing, were based on this subsampled dataset.

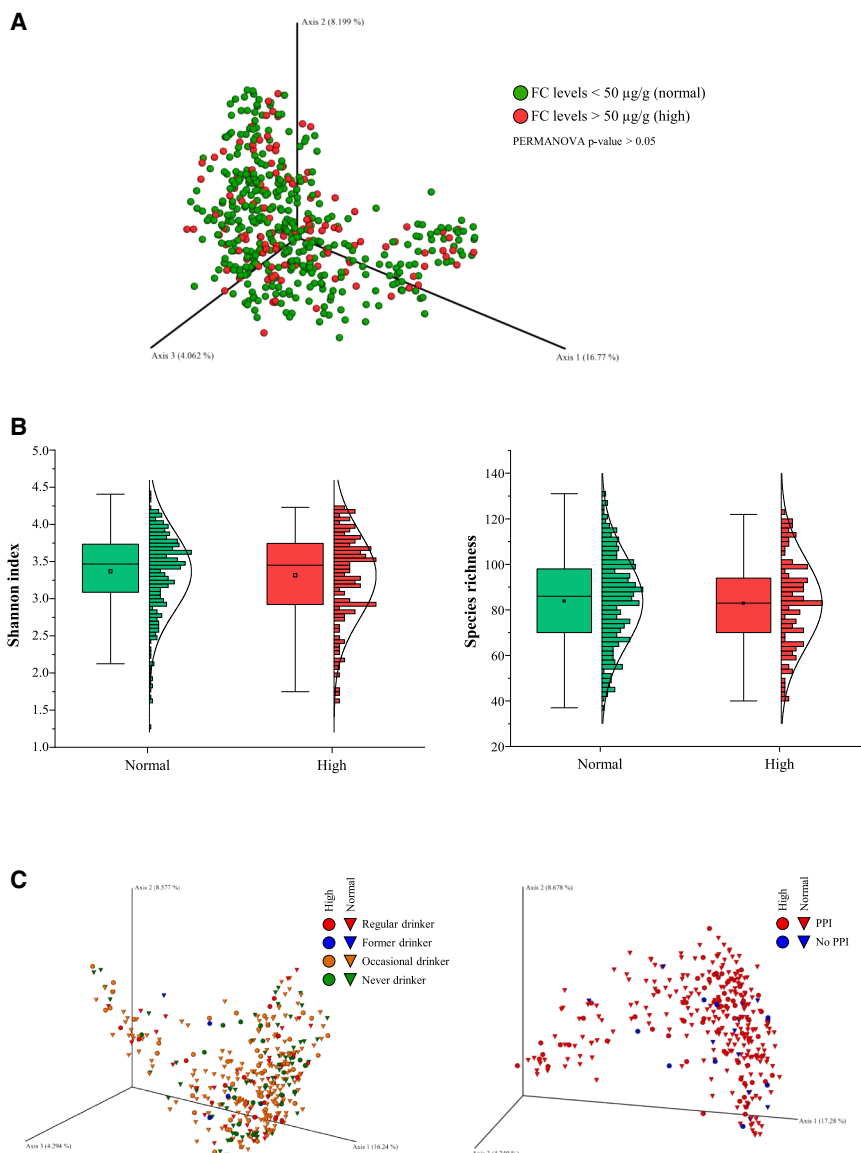


**Figure 2. Microbial biodiversity in the two groups of FC levels**

(A) Principal coordinates analysis (PCoA) plot based on Bray-Curtis dissimilarity at the species level, illustrating beta-diversity of microbial communities in samples grouped by FC levels. Group differences were assessed by PERMANOVA.

(B) Box-and-whisker plots representing alpha-diversity metrics (species richness and Shannon index) for each FC level group. The x axis represents the FC level groups, while the y axis indicates diversity values. Boxes represent the interquartile range ([IQR], 25th–75th percentiles), whiskers extend to 1.5 times the IQR, and the line within the boxes indicates the median. Squares denote the group averages.

(C) PCoA plot based on Bray-Curtis dissimilarity showing the possible impact of significant factors such as alcohol consumption (left) and chronic PPI use (right) on the microbiota composition in the two groups (normal and high) of FC levels. Significance was assessed by the Adonis implementation of PERMANOVA. See also Figure S1; Tables S1 and S2.

Similarities) and PERMANOVA,  $p$  values  $>0.05$ ), confirming the overall diversity pattern observed in the whole cohort.

To evaluate the impact of significant factors, such as alcohol consumption and chronic PPI use, on gut microbiota composition, specific analyses through the Adonis implementation of PERMANOVA were performed on the subset of samples with these metadata. No relation between these two factors and the microbiota composition was detected (Adonis  $p$  value  $>0.05$ ) (Figure 2C).

### Correlation between specific taxa and microbial metabolic pathways with calprotectin levels

A combination of independent statistical

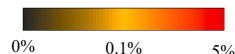
The taxonomic profiles obtained for each sample were used to evaluate the microbial biodiversity between the normal and high groups (Table S2). In detail, a specific analysis of the beta-diversity, calculated through the Bray-Curtis dissimilarity index based on the bacterial profile at the species level, revealed no significant inter-individual variation between the analyzed groups (PERMANOVA (Permutational Multivariate Analysis of Variance)  $p$  value  $>0.05$ ) (Figure 2A). The alpha-diversity, calculated by species richness and Shannon indices, also showed no significant differences between the normal and high groups (Mann-Whitney U test  $p$  values  $>0.05$ ) (Figure 2B) and showed a remarkable heterogeneity in terms of bacterial complexity within the two groups. An additional analysis on a subset of samples with a higher FC threshold of 150  $\mu\text{g/g}$  ( $n = 27$ ) did not reveal significant differences in the main alpha- or beta-diversity metrics (Figure S1, species richness and Shannon index, Mann-Whitney U test  $p$  values  $>0.05$ ; ANOSIM (Analysis of

approaches, relative difference in abundance, Mann-Whitney U test, PLS-DA (partial least squares discriminant analysis), and RG (group indicators) was applied to evaluate bacterial species specifically associated with normal or high levels of FC. To ensure robustness in the results, we considered only microbial taxa that met significance in at least two statistical approaches. For abundance-based analyses, we selected taxa with relative differences greater than 100% or less than  $-50\%$  and a  $p$  value  $<0.05$  as found by the Mann-Whitney U test. For prevalence and group discrimination, we included taxa with VIP scores above 1 (as calculated by PLS-DA) and those meeting statistical significance ( $p$  value  $<0.05$ ) in the RG (group indicators) analysis. A total of 28 bacterial taxa were identified, of which 4 were associated with the normal group and 24 with the high FC group (Figure 3A).

To further explore the relationship between FC levels and the bacterial taxa, a Spearman correlation analysis was performed to correlate the FC values of each sample with its corresponding

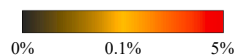
A

| Phylum            | Species                               | Relative abundance FC<br>< 50 µg/g samples | Relative abundance FC<br>> 50 µg/g samples | Relative difference | Mann-Whitney U test<br>p-value | VIP-score | RG (Group Indicators)<br>p-value |
|-------------------|---------------------------------------|--------------------------------------------|--------------------------------------------|---------------------|--------------------------------|-----------|----------------------------------|
| Bacteroidota      | <i>Coprobacter</i> unknown_species    | 0.022%                                     | 0.008%                                     | 184.20%             | 0.0380                         | 1.90      | 0.020                            |
| Bacteroidota      | <i>Bacteroides intestinalis</i>       | 0.774%                                     | 0.273%                                     | 183.09%             | -                              | 1.93      | 0.011                            |
| Bacteroidota      | <i>Prevotella rara</i>                | 0.741%                                     | 0.317%                                     | 134.11%             | 0.0170                         | 1.44      | 0.048                            |
| Bacteroidota      | <i>Prevotella marshallensis</i>       | 0.641%                                     | 0.294%                                     | 118.30%             | 0.0360                         | 1.33      | -                                |
| Bacteroidota      | <i>Odoribacter</i> unknown_species    | 0.021%                                     | 0.042%                                     | -50.22%             | 0.0010                         | 2.99      | 0.003                            |
| Bacillota         | <i>Ruminococcus faecis</i>            | 0.078%                                     | 0.159%                                     | -51.00%             | 0.0360                         | 2.57      | 0.013                            |
| Bacillota         | <i>Ruminococcus callidus</i>          | 0.024%                                     | 0.048%                                     | -51.05%             | 0.0020                         | 1.93      | 0.044                            |
| Pseudomonadota    | <i>Haemophilus parainfluenzae</i>     | 0.066%                                     | 0.138%                                     | -51.97%             | 0.0180                         | 1.98      | 0.040                            |
| Bacillota         | <i>Veillonella rogosa</i>             | 0.014%                                     | 0.035%                                     | -59.20%             | 0.0060                         | 1.71      | -                                |
| Bacillota         | <i>Veillonella parvula</i>            | 0.028%                                     | 0.095%                                     | -70.14%             | 0.0130                         | 2.07      | 0.024                            |
| Bacillota         | <i>Veillonella atypica</i>            | 0.012%                                     | 0.042%                                     | -71.10%             | 0.0190                         | 1.57      | -                                |
| Pseudomonadota    | <i>Haemophilus</i> unknown_species    | 0.008%                                     | 0.033%                                     | -75.34%             | 0.0040                         | 2.46      | 0.018                            |
| Bacillota         | <i>Streptococcus</i> unknown_species  | 0.006%                                     | 0.026%                                     | -76.04%             | 0.0004                         | 2.87      | 0.013                            |
| Bacteroidetes     | <i>Bacteroides stercorisoris</i>      | 0.004%                                     | 0.022%                                     | -83.55%             | -                              | 1.80      | 0.038                            |
| Pseudomonadota    | <i>Klebsiella variicola</i>           | 0.002%                                     | 0.014%                                     | -83.87%             | 0.0160                         | 1.27      | -                                |
| Bacillota         | <i>Holdemania filiformis</i>          | 0.000%                                     | 0.003%                                     | -88.14%             | -                              | 1.72      | 0.050                            |
| Bacillota         | <i>Streptococcus salivarius</i>       | 0.012%                                     | 0.104%                                     | -88.46%             | 0.0000                         | 4.45      | 0.001                            |
| Bacillota         | <i>Catenibacillus</i> unknown_species | 0.000%                                     | 0.005%                                     | -92.04%             | 0.0150                         | 2.16      | 0.023                            |
| Bacillota         | <i>Streptococcus parasanguis</i>      | 0.002%                                     | 0.054%                                     | -96.72%             | 0.0000                         | 5.25      | 0.001                            |
| Methanobacteriota | <i>Methanobrevibacter smithii</i>     | 0.001%                                     | 0.115%                                     | -99.29%             | 0.0030                         | 2.15      | 0.004                            |
| Pseudomonadota    | <i>Citrobacter</i> unknown_species    | 0%                                         | 0.002%                                     | -100.00%            | 0.0110                         | 2.08      | -                                |
| Bacillota         | <i>Enorma massiliensis</i>            | 0%                                         | 0.003%                                     | -100.00%            | 0.0110                         | 2.21      | -                                |
| Pseudomonadota    | <i>Escherichia albertii</i>           | 0%                                         | 0.003%                                     | -100.00%            | 0.0110                         | 2.16      | -                                |
| Pseudomonadota    | <i>Haemophilus pittmaniae</i>         | 0%                                         | 0.015%                                     | -100.00%            | 0.0110                         | 1.80      | -                                |
| Pseudomonadota    | <i>Klebsiella oxytoca</i>             | 0%                                         | 0.004%                                     | -100.00%            | 0.0110                         | 2.04      | 0.049                            |
| Bacillota         | <i>Lactobacillus gasseri</i>          | 0%                                         | 0.006%                                     | -100.00%            | 0.0110                         | 2.20      | 0.050                            |
| Bacillota         | <i>Leuconostoc lactis</i>             | 0%                                         | 0.024%                                     | -100.00%            | 0.0020                         | 2.43      | 0.014                            |
| Pseudomonadota    | <i>Proteus mirabilis</i>              | 0%                                         | 0.006%                                     | -100.00%            | 0.0110                         | 1.79      | -                                |



B

| Enzyme activities                    | Rhea  | Relative abundance FC<br>< 50 µg/g samples | Relative abundance FC<br>> 50 µg/g samples | Relative difference | Mann-Whitney U test<br>p-value | VIP-score | RG (Group Indicators)<br>p-value |
|--------------------------------------|-------|--------------------------------------------|--------------------------------------------|---------------------|--------------------------------|-----------|----------------------------------|
| Inositol-polyphosphate 5-phosphatase | 11392 | 0.0000022%                                 | 0%                                         | +500.00%            | -                              | 1.14      | -                                |
| Cysteine dioxygenase                 | 20441 | 0.0000207%                                 | 0.0000088%                                 | 134.19%             | -                              | 1.41      | -                                |
| 3',5'-Cyclic-GMP phosphodiesterase   | 16957 | 0.0001237%                                 | 0.0000844%                                 | 46.61%              | 0.041                          | 1.29      | -                                |
| Glucuronosyltransferase              | 21032 | 0.0002182%                                 | 0.0001619%                                 | 34.76%              | -                              | 1.85      | 0.044                            |
| Alcohol dehydrogenase                | 10736 | 0.0555357%                                 | 0.0484151%                                 | 14.71%              | 0.005                          | 2.01      | 0.044                            |
| Alcohol dehydrogenase                | 25290 | 0.0310081%                                 | 0.0271280%                                 | 14.30%              | 0.036                          | 1.66      | -                                |
| Geranylgeranyl diphosphate synthase  | 17653 | 0.0090697%                                 | 0.0080481%                                 | 12.69%              | 0.036                          | 2.17      | 0.028                            |
| Acetate-CoA ligase (ADP-forming)     | 15081 | 0.0114779%                                 | 0.0102438%                                 | 12.05%              | 0.035                          | 1.78      | -                                |
| Alcohol dehydrogenase                | 10740 | 0.0299785%                                 | 0.0274909%                                 | 9.05%               | 0.021                          | 1.53      | -                                |
| Argininosuccinate synthase           | 10932 | 0.0872908%                                 | 0.0836034%                                 | 4.41%               | -                              | 1.08      | -                                |
| Prephenate dehydratase               | 21648 | 0.1471638%                                 | 0.1410458%                                 | 4.34%               | 0.027                          | 2.21      | 0.021                            |
| Methionine synthase                  | 11172 | 0.3575137%                                 | 0.3679845%                                 | -2.85%              | 0.019                          | 2.01      | 0.046                            |
| Dihydrofolate reductase              | 15009 | 0.0456573%                                 | 0.0483239%                                 | -5.52%              | 0.02                           | 3.13      | 0.004                            |
| Acetyl-CoA hydrolase                 | 20289 | 0.0528096%                                 | 0.0565538%                                 | -6.62%              | -                              | 2.04      | 0.046                            |
| Phenylalanine 4-monoxygenase         | 20273 | 0.0000270%                                 | 0.0000587%                                 | -53.98%             | -                              | 1.94      | -                                |
| Heme oxygenase (mycobilin-producing) | 52236 | 0.0000007%                                 | 0.0000037%                                 | -81.22%             | -                              | 1.50      | -                                |
| Heme oxygenase (mycobilin-producing) | 52232 | 0.0000007%                                 | 0.0000037%                                 | -81.22%             | -                              | 1.50      | -                                |
| N(1)-acetylpolymine oxidase          | 25804 | 0.0000004%                                 | 0.0000025%                                 | -85.30%             | -                              | 1.42      | -                                |
| N(1)-acetylpolymine oxidase          | 25812 | 0.0000004%                                 | 0.0000025%                                 | -85.30%             | -                              | 1.42      | -                                |
| N(1)-acetylpolymine oxidase          | 25808 | 0.0000004%                                 | 0.0000025%                                 | -85.30%             | -                              | 1.42      | -                                |
| N(1)-acetylpolymine oxidase          | 25800 | 0.0000004%                                 | 0.0000025%                                 | -85.30%             | -                              | 1.42      | -                                |
| Nitrilase                            | 45776 | 0.0000003%                                 | 0.0000026%                                 | -86.67%             | -                              | 1.42      | -                                |
| Naphthalene 1,2-dioxygenase          | 19173 | 0.0000017%                                 | 0.0000155%                                 | -89.26%             | 0.006                          | 2.86      | 0.004                            |
| Tannase                              | 16365 | 0%                                         | 0.0000013%                                 | -100.00%            | -                              | 1.85      | -                                |



(legend on next page)

**Table 2. Spearman's rank correlation between fecal calprotectin levels and bacterial relative abundance**

| Species                              | Correlation | P.adjusted.BH |
|--------------------------------------|-------------|---------------|
| <i>Lachnospira</i> unknown_species   | −0.1511     | 0.011         |
| <i>Hungatella</i> unknown_species    | −0.1380     | 0.028         |
| <i>Clostridium</i> unknown_species   | −0.1348     | 0.034         |
| <i>Roseburia hominis</i>             | −0.1325     | 0.039         |
| <i>Escherichia coli</i>              | 0.1423      | 0.021         |
| <i>Streptococcus</i> unknown_species | 0.1452      | 0.017         |
| <i>Odoribacter</i> unknown_species   | 0.1701      | 0.002         |
| <i>Butyricimonas</i> unknown_species | 0.1922      | 0.000         |
| <i>Streptococcus salivarius</i>      | 0.2301      | 0.000         |
| <i>Streptococcus parasanguinis</i>   | 0.2417      | 0.000         |

taxonomic profile. This analysis allowed the identification of 10 taxa with significant correlations ( $p$  value  $<0.05$ ), of which 4 negatively associated with low levels of the fecal marker (i.e., *Lachnospira*, *Hungatella*, *Clostridium*, and *Roseburia hominis*), and 6 positively associated with high FC levels (i.e., *Escherichia coli*, *Streptococcus*, *Odoribacter*, *Butyricimonas*, *Streptococcus salivarius*, and *Streptococcus parasanguinis*) (Table 2). These results suggest possible correlations between the presence of specific bacterial taxa and FC.

The shotgun metagenomics approach also allowed the prediction and analysis of the functional potential of the microbiome. For functional profiling, only samples with at least 5,000,000 reads were retained after quality filtering and removal of *Homo sapiens* reads. Accordingly, a total of 397 samples from the Parma Microbiota project were included in this analysis, with 308 belonging to the normal group and 89 to the high-FC group (Table S3). Microbial enzymatic activities were annotated using the Rhea database and analyzed to find possible associations with normal or high FC levels, applying the same statistical approach used for taxonomic analysis (see above). Overall, this process yielded a total of 422,374,094 reads assigned to Rhea-based reactions, with an average of  $820,144 \pm 127,437$  reads per sample (Table S3). In brief, we combined multiple statistical methods, including relative abundance differences, Mann-Whitney U test, PLS-DA, and RG indicators, to ensure a robust choice of significant enzymatic activities. Given the complexity of microbial metabolic networks, the analysis was restricted to 184 Rhea enzyme reactions, selected based on their role in human health, following an extensive literature review (Table S4).

A total of 24 microbially driven enzymatic activities were identified, of which 13 associated with the normal group and 11 with the high FC group (Figure 3B). Additionally, the relationship between FC levels and microbial functional capabilities was explored through a Spearman correlation analysis to correlate the FC values of each sample with its corresponding enzymatic

activities (Table 3). This analysis allowed the identification of 28 metabolic enzymatic activities with significant correlations ( $p$  value  $<0.05$ ), of which 12 negatively correlated and 16 positively correlated with FC values. Positive correlation suggests a potential association with intestinal inflammation and/or an adaptation of the intestinal microbiome to the secretion of FC (Table 3). These findings suggest a potential link between specific metabolic activities of the gut microbiome and FC levels.

## DISCUSSION

FC is a widely recognized marker of intestinal inflammation, highly sensitive but not very specific for IBD. On the other hand, specific fecal microbial taxa have been suggested as novel biomarkers. However, most studies did not consider confounding factors that could influence FC levels and/or microbial composition, such as host extrinsic (e.g., drug intake or previous infectious diseases) and host intrinsic (e.g., chronic diseases or psychopathologies) factors.<sup>20,21</sup>

In this study, the connection between FC levels and fecal microbiota composition was evaluated in a cohort of northern Italy subjects, standing for the dataset of the Parma Microbiota project (<https://www.microbiomeresearchhub.com/parmamicrobiota>), who did not have symptoms referable to ongoing intestinal pathologies. The aim of the study is to find early microbial patterns associated with elevated FC levels potentially related to an intestinal inflammatory status, excluding the effect of established inflammatory diseases and discriminating against the influence of possible confounding factors.

The median FC value in the 515 enrolled individuals ( $23.6 \mu\text{g/g}$ ) is in line with the known FC concentration in healthy individuals, which ranges between 10 and  $50 \mu\text{g/g}$  depending on the study cohorts and the measurement methods used.<sup>4</sup> Most of the enrolled individuals of the Parma Microbiota project have FC levels below the normal threshold of  $50 \mu\text{g/g}$ , confirming the good gut conditions of the study population. However, over 20% of the individuals have FC levels above  $50 \mu\text{g/g}$ , with a marked heterogeneity in FC levels among asymptomatic individuals. This observation may be consistent with a subclinical alteration in intestinal inflammatory status at the time of enrollment, although larger cohorts, longitudinal follow-up, and clinical validation will be needed to confirm this hypothesis. Among the considered confounding factors, only alcohol consumption and, more strongly, the chronic use of PPIs are correlated with FC levels  $>50 \mu\text{g/g}$ . Among these, the use of PPI is a known factor capable of increasing FC levels,<sup>6</sup> but it does not impact the composition of the microbiota, as evidenced by a multivariate analysis considering all three factors (Figure 2C).

The microbiota composition analysis did not reveal significant differences in beta-diversity or alpha-diversity between the groups with normal and high FC levels, consistent with the results obtained in a cohort of older individuals.<sup>13</sup> Also, an additional analysis using a more stringent FC threshold of  $150 \mu\text{g/g}$

**Figure 3. Differences in microbiota composition and metabolic pathways associated with FC**

Tables summarizing bacterial species (A) and microbial enzymatic activities (B) identified as significantly associated with FC levels through a combination of independent statistical approaches, including relative difference in abundance, Mann-Whitney U test, partial least squares discriminant analysis (PLS-DA), and RG (group indicators). The significance of bacterial taxa and enzymatic activities in at least two approaches were considered robustly associated with either normal or high FC levels. See also Tables S3 and S4.

**Table 3. Spearman's rank correlation between fecal calprotectin levels and bacterial enzymatic activities**

| Enzyme class                                    | Rhea  | Correlation | P.adjusted.BH |
|-------------------------------------------------|-------|-------------|---------------|
| Alcohol dehydrogenase                           | 10736 | −0.1774     | 0.0014        |
| 3',5'-Cyclic-GMP phosphodiesterase              | 16957 | −0.1647     | 0.0034        |
| Hydroxymethylglutaryl-CoA reductase (NADPH)     | 15989 | −0.1528     | 0.0073        |
| Alcohol dehydrogenase                           | 25290 | −0.1476     | 0.0101        |
| Geranylgeranyl diphosphate synthase             | 17653 | −0.1453     | 0.0116        |
| Alcohol dehydrogenase                           | 10740 | −0.1417     | 0.0142        |
| Glutamate N-acetyltransferase                   | 15349 | −0.1416     | 0.0143        |
| Cysteine dioxygenase                            | 20441 | −0.137      | 0.0186        |
| Propionate CoA-transferase                      | 23520 | −0.1329     | 0.0233        |
| Prephenate dehydratase                          | 21648 | −0.1203     | 0.0447        |
| Biotin-[biotin carboxyl-carrier protein] ligase | 11756 | −0.1185     | 0.0489        |
| Isoleucine-tRNA ligase                          | 11060 | −0.1181     | 0.0498        |
| Glutamate dehydrogenase                         | 15133 | 0.1183      | 0.0493        |
| Magnesium transporter                           | 29827 | 0.1189      | 0.0478        |
| Homoserine dehydrogenase                        | 15757 | 0.1325      | 0.0239        |
| Acetyl-CoA hydrolase                            | 20289 | 0.1326      | 0.0237        |
| Arylesterase                                    | 17309 | 0.1334      | 0.0227        |
| Cystathionine gamma-lyase                       | 24931 | 0.1367      | 0.0189        |
| Adenylate cyclase                               | 15389 | 0.1408      | 0.015         |
| Methionine synthase                             | 11172 | 0.1516      | 0.0079        |
| Phosphoserine phosphatase                       | 24873 | 0.1521      | 0.0076        |
| Phosphoserine phosphatase                       | 21208 | 0.1521      | 0.0076        |
| Acetylornithine deacetylase                     | 15941 | 0.1557      | 0.0061        |
| Nitrite reductase [NAD(P)H]                     | 24628 | 0.1722      | 0.002         |
| Arsenate reductase (glutathione/glutaredoxin)   | 22016 | 0.1771      | 0.0014        |
| NADPH:quinone reductase                         | 14269 | 0.1854      | 0.0008        |
| 3(or 17)beta-hydroxysteroid dehydrogenase       | 14981 | 0.1916      | 0.0005        |
| 3(or 17)beta-hydroxysteroid dehydrogenase       | 14929 | 0.1928      | 0.0004        |

did not reveal significant differences in the main diversity metrics, further supporting the overall biodiversity pattern observed with the 50 µg/g stratification. Moreover, while previous studies have suggested that calprotectin may exert antimicrobial effects by sequestering essential metal ions,<sup>4,5</sup> our data did not show a significant impact on overall bacterial cell abundance. These results are consistent with the absence of symptoms attributable to overt intestinal pathologies, pointing to a situation in which substantial shifts in microbiota overall abundance and diversity may not be yet apparent.<sup>22</sup>

However, by focusing on the taxonomic profiles, we identified specific bacterial taxa that were significantly associated with FC levels. In detail, individuals with normal FC levels revealed a higher abundance of bacterial taxa commonly associated with

gut health and anti-inflammatory effects, such as *Bacteroides intestinalis*. These species are known producers of butyrate, a short-chain fatty acid that plays a crucial role in maintaining intestinal barrier integrity and down-regulating inflammation.<sup>23</sup> In particular, butyrate promotes the differentiation of regulatory T cells, which are essential for immune tolerance, the suppression of inflammatory responses,<sup>24,25</sup> and the polarization of macrophages toward the anti-inflammatory M2 phenotype.<sup>26</sup> In contrast, individuals with high FC levels were associated with a more diverse array of bacterial taxa, many of which are opportunistic pathogens or known to thrive in dysbiotic environments, such as *Haemophilus parainfluenzae*, *Klebsiella variicola*, *Escherichia albertii*, *Streptococcus salivarius*, and *Proteus mirabilis*.<sup>13,27–29</sup> Many of these taxa belong to the Pseudomonadota and Bacillota phyla, often associated with infections and inflammatory conditions.<sup>30,31</sup> For example, the increased abundance of *Klebsiella* and *Escherichia* in individuals with high FC levels could reflect an imbalance in the microbiota composition, thus reflecting altered intestinal conditions associated with elevated FC levels.<sup>32,33</sup> Moreover, the presence of these taxa could also coincide with a compromised gut barrier, a condition referred as leaky gut,<sup>34,35</sup> which may facilitate the translocation of bacterial metabolites, such as lipopolysaccharide, into the systemic circulation, thereby triggering immune activation and inflammation.<sup>34,35</sup>

Spearman correlation analyses further support the association between specific taxa and FC levels. *Lachnospira*, *Hungatella*, *Clostridium*, and *Roseburia hominis* were negatively correlated with FC levels, and some of these bacterial taxa, particularly those belonging to the genera *Hungatella* and *Roseburia*, are butyrate producers.<sup>36,37</sup> Conversely, taxa such as *Escherichia coli*, *Streptococcus salivarius*, *Streptococcus parasanguinis*, *Butyrivibrio*, and *Odoribacter*, indicative of altered gut microbiota composition,<sup>38,39</sup> showed positive correlations with FC levels, confirming the results presented in Figure 3A.

In addition to taxonomic differences, functional analyses of microbial enzymatic activities offered further insights into the possible relationship between microbiota and intestinal FC levels. Several microbially driven metabolic pathways were significantly enriched in the group with elevated levels of FC, such as polyamine degradation (RHEA: 25804, 25812, 25808, and 25800). High levels of polyamines like spermidine and spermine are associated with epithelial damage, cell proliferation, and inflammatory responses.<sup>40,41</sup> Therefore, the observed increase in bacterial capacity to degrade polyamines in individuals with elevated FC may be consistent with microbial responses associated with a pro-inflammatory gut environment. Conversely, the group with normal levels of FC showed a possible association with several enzymatic activities, such as inositol-polyphosphate 5-phosphatase (RHEA: 11392) and cysteine dioxygenase (RHEA: 20441). Inositol-polyphosphate 5-phosphatase plays a crucial role in phosphatidylinositol metabolism, which is fundamental for cellular signaling pathways involved in inflammation and intestinal barrier regulation. This enzyme modulates key signaling cascades that influence epithelial integrity and immune responses, potentially contributing to a more stable intestinal environment in individuals with lower FC levels.<sup>42</sup> In turn, cysteine dioxygenase catalyzes the conversion of cysteine into cysteine sulfinic acid, a precursor of taurine or of

3-sulfinylpyruvate, and hence of pyruvate and sulfite. Cysteine is needed for the synthesis of glutathione, a critical antioxidant involved in cellular protection against oxidative stress and inflammation,<sup>43</sup> but the amino acid can also have direct cytotoxic effects when present in excessive quantity.<sup>44</sup> Conversely, taurine is a known protein stabilizer.<sup>45</sup> Therefore, cysteine catabolism may have favorable effects for both the intestinal cells and the microbiota, since cysteine dioxygenase limits the availability of cysteine and, at the same time, provides a metabolic source of sulfite and taurine. Spearman correlation analysis further revealed specific enzymatic activities correlated with low FC, such as cGMP degradation (RHEA: 16957) and propionate production (RHEA: 23520), which contribute to gut homeostasis supporting intestinal barrier integrity<sup>46,47</sup> and anti-inflammatory status,<sup>48,49</sup> respectively. Another noteworthy finding of our study is the positive correlation between FC levels and bacterial magnesium transport (RHEA: 29827). Calprotectin is known to sequester metals such as magnesium to limit bacterial growth, but the microbiota might compensate by upregulating magnesium transport systems to counteract this limitation. This hypothesis is further supported by flow cytometry data that showed no significant differences in terms of bacterial load seen in high FC group. This may be consistent again with microbial responses associated with high FC levels, potentially involving compensatory strategies related to ionic homeostasis.<sup>50</sup>

Overall, these results support an association between gut microbiota composition, microbial functional potential, and FC levels. The observed taxonomic and functional differences involve microbial features plausibly linked to intestinal homeostasis, short-chain fatty acid metabolism, barrier integrity, and microbial responses to altered intestinal conditions. However, the cross-sectional design of the present study does not allow us to determine whether the observed taxonomic and functional features are a consequence of altered intestinal inflammatory status, contribute to it, or simply co-exist with elevated FC levels. Moreover, the functional associations observed here should be interpreted with caution since they reflect microbial functional potential inferred from metagenomic annotation, rather than providing direct evidence of metabolic activity. Future studies should explore the role of microbial metabolic pathways in calprotectin modulation and their impact on gut health. Despite the absence of significant differences in microbiota diversity, the enrichment of beneficial butyrate-producing taxa in individuals with normal FC levels and the higher abundance of opportunistic pathogens in those with elevated FC levels are consistent with biologically meaningful differences between the two groups. If confirmed in longitudinal and clinically validated studies, the taxonomic and functional microbial features identified here may represent candidate microbial patterns associated with elevated FC levels. Potential biomarker application will require external validation, assessment of reproducibility across populations, and correlation with clinically meaningful outcomes.

### Limitations of the study

Despite the strengths of the present work, some limitations should be considered. The threshold of 50 µg/g was used as a stratification criterion corresponding to the upper limit of the range commonly considered normal in healthy adults, rather than as a

diagnostic threshold for clinically established intestinal inflammation. Therefore, the interpretation of high FC levels in apparently asymptomatic individuals as indicative of intestinal inflammatory status should be regarded as hypothesis generating, since it remains unclear whether these values reflect a stable subclinical alteration in intestinal inflammatory status, transient fluctuations, or an increased risk of future inflammatory conditions. In addition, although several potential confounding factors were considered in the present study, microbiota-FC associations should be interpreted within a broader host and lifestyle context, including dietary habits, which may well contribute to inter-individual variability.

While taxonomic profiling was performed using a widely adopted shallow-shotgun strategy,<sup>18</sup> with read-depth normalization and subsampling to ensure comparability across samples, deeper sequencing may further improve the characterization of low-abundance taxa and microbial functions. Moreover, the functional profiles inferred from metagenomic annotation reflect microbial functional potential rather than direct metabolic activity. Therefore, complementary metabolomic and/or transcriptomic approaches will be necessary to validate the mechanistic significance of the associations observed here.<sup>51,52</sup>

Finally, the cross-sectional and single-center design does not allow causal inference and may limit the general relevance of the results. Future multicenter studies including external validation cohorts, repeated FC measurements, and longitudinal follow-up will be essential to confirm the biological and clinical meaning of the microbial signatures identified in this work.

### RESOURCE AVAILABILITY

#### Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Ovidio Bussolati ([ovidio.bussolati@unipr.it](mailto:ovidio.bussolati@unipr.it)).

#### Materials availability

This study did not generate any new unique reagents.

#### Data and code availability

- Raw Italian shotgun metagenomic sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under NCBI BioProject PRJNA1046438.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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### AUTHOR CONTRIBUTIONS

Conceptualization, O.B. and M.V.; software, G.A.L.; formal analysis, G.T. and L.M.; investigation, G.T., G.L., C.U., M.G.B., and M.C.; data curation, L.M.; writing – original draft, G.T. and L.M.; writing – review and editing, C.M.,

S.K., F.G., R.A., O.B., and M.V.; visualization, G.T. and L.M.; supervision, F.T. and O.B.; project administration, M.V.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

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

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Measurement of fecal calprotectin (FC)
  - Evaluation of bacterial cell density by flow cytometry
  - Taxonomic classification
  - Functional prediction
- QUANTIFICATION AND STATISTICAL ANALYSIS

## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE                                        | SOURCE                          | IDENTIFIER                    |
|------------------------------------------------------------|---------------------------------|-------------------------------|
| <b>Biological samples</b>                                  |                                 |                               |
| Human fecal samples ( $n = 515$ ), collected in this study | This study                      | N/A                           |
| <b>Chemicals, peptides, and recombinant proteins</b>       |                                 |                               |
| Inactivating liquid                                        | Zymo Research, USA              | DNA/RNA Shield                |
| <b>Critical commercial assays</b>                          |                                 |                               |
| Sample Preparation System Stool                            | DRG Instruments GmbH, Germany   | Cat#EIA-5674                  |
| Calprotectin (MRP 8/14) Stool ELISA                        | DRG Instruments GmbH, Germany   | Cat#EIA-5415                  |
| SYBR®Green I                                               | ThermoFisher Scientific, USA    | Cat#S7567                     |
| QIAamp DNA Stool Mini kit                                  | QIAGEN, Hilden, Germany         | Cat#51604                     |
| <b>Deposited data</b>                                      |                                 |                               |
| Raw Italian shotgun metagenomic sequencing data            | This study                      | accession number PRJNA1046438 |
| <b>Software and algorithms</b>                             |                                 |                               |
| SPSS (v25)                                                 | IBM                             | Version 25                    |
| R (v4.1.2)                                                 | The R Foundation                | Version 4.1.2                 |
| METAnnotatorX2                                             | Milani et al. <sup>16</sup>     | N/A                           |
| Attune NxT                                                 | Thermo Fisher Scientific        | Model: AFC2                   |
| MegaBLAST                                                  | Vandeputte et al. <sup>53</sup> | N/A                           |
| RAPSearch2                                                 | Chen et al. <sup>54</sup>       | N/A                           |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

A total of 515 stool samples have been collected starting from September 2021 in the frame of Parma Microbiota project (Ethics Committee of the Area Vasta Emilia Nord, ID 1107/2020/TESS/UNIPR), a cross-sectional project that does not involve the use of drugs or medical devices, but only the administration of questionnaires and the performance of non-diagnostic analyses on biological materials. The study was conducted on volunteers with no known intestinal pathologies living in Parma area (Emilia-Romagna, Italy), after obtaining their written informed consent for participation in the study and the processing of personal data. Inclusion criteria were age  $\geq 18$  years; residence in Parma city or hinterland; reported good health conditions with no known intestinal pathology. Exclusion criteria were personal history of IBD, other chronic inflammatory or gastrointestinal diseases; gastrointestinal (diarrhea, abdominal pain, constipation, melena, jaundice, dyspepsia) or systemic symptoms/signs (fever, arthralgias) suggestive of IBD; prolonged antibiotic therapies in the 20 days prior to enrollment; severe extraintestinal diseases with a poor prognosis; active malignant neoplasms and/or active chemo- or radiotherapy and/or positive history of malignant neoplasm; inability to understand and make decisions; severe disability with limitation of activities; documented positivity for SARS-CoV-2 in the 30 days prior to evaluation.

Each participant was provided with instructions to collect a small amount of stool (approximately 6–10 g), to divide it into two containers, to store it at room temperature away from heat sources, and to deliver it to the laboratory. One container (for microbiome studies) contained an inactivating liquid (Zymo Research, USA) to block bacterial replication, while the other was empty for fecal calprotectin assay, which requires a sample without any addition that might interfere with the analysis.<sup>6</sup> Finally, each subject was asked to complete a questionnaire to collect metadata.

## METHOD DETAILS

## Measurement of fecal calprotectin (FC)

The samples were slowly thawed to avoid the burst of neutrophils and a mix of three extracts from the same sample was collected with a specific device (Sample Preparation System Stool, DRG Instruments GmbH, Germany) to obtain a representative amount of 15 mg for each sample and suspended in an extraction buffer. Calprotectin was quantified through an ELISA assay, following the manufacturer's instructions (Calprotectin (MRP 8/14) Stool ELISA, DRG Instruments GmbH, Germany), based on a two-site sandwich approach with monoclonal antibodies that bind to human calprotectin. In each analytical session, a standard curve and two internal controls with known FC concentrations were also performed. Results were expressed in  $\mu\text{g/g}$  of stool. The test has a limit of quantification of 4.767 ng/mL, corresponding to 10  $\mu\text{g/g}$  of stool, which is why all values obtained below this threshold were considered as 10  $\mu\text{g/g}$ .

### Evaluation of bacterial cell density by flow cytometry

For total cell counts, 0.2 g of each fecal sample was processed and diluted 1:100,000 in phosphate buffered saline (PBS). Subsequently, 1 mL of the obtained bacterial cell suspension was stained with 1  $\mu$ L of SYBR® Green I (ThermoFisher Scientific, USA), previously diluted 1:100 in dimethyl sulfoxide (Sigma, Germany), vortex-mixed, and incubated at 37°C in the dark for at least 15 min before measurement. Count experiments were performed using an Attune NxT flow cytometry (ThermoFisher Scientific, Waltham, MA, USA) equipped with a blue laser set at 50 mW and tuned at an excitation wavelength of 488 nm. Multiparametric analyses included forward scatter (FSC), side scatter (SSC), and SYBR Green I fluorescence detected on the BL1 530/30 nm optical detector. Bacterial events were first identified as SYBR Green I-positive above the BL1 threshold to exclude non-fluorescent debris and were subsequently refined on the FSC/SSC density plot to remove residual background events, as previously described.<sup>53</sup> The gating strategy used for bacterial enumeration is illustrated by representative plots in Figure S2. Bacterial counts were corrected for the dilution factor and expressed as cells/mL of fecal suspension. Data were analyzed using Attune NxT flow cytometry software.

### Taxonomic classification

The extraction of bacterial DNA was performed using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions (<https://www.qiagen.com/be>). The collected samples were then sequenced through Illumina shotgun metagenomic sequencing. Briefly, DNA libraries were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol, starting from 1 ng of input DNA per sample. The extracted DNA underwent fragmentation, adapter ligation, and amplification. The resulting libraries were pooled at equimolar concentrations, denatured, and diluted to a final loading concentration of 1.5 pM. Sequencing was performed on an Illumina NextSeq 2000 instrument (Illumina, San Diego, CA, USA) using a 2  $\times$  150 bp paired-end High Output sequencing kit, with a 1% PhiX control library spike-in. Raw reads were processed using the METAnnotatorX2 pipeline.<sup>16,17</sup> Filtering was performed using the standard pipeline settings, including removal of reads shorter than 100 bp, reads with a quality score below 20, and *Homo sapiens* reads. Taxonomic classification was performed on 100,000 reads per sample using MegaBLAST<sup>54</sup> against a manually curated and pre-processed database of genomes retrieved from the National Center for Biotechnology Information, following the METAnnotatorX2 manual,<sup>16</sup> with standard settings including a minimum query coverage of 50% and a 94% identity threshold for species-level assignment. Subsampling to 100,000 reads per sample was applied to normalize read depth across samples prior to taxonomic assignment, in accordance with a shallow-shotgun metagenomic strategy aimed at ensuring robust and comparable taxonomic profiling of microbial community composition.<sup>18,19</sup>

### Functional prediction

Microbial functional profiling of the sequenced reads was performed with the METAnnotatorX2 bioinformatic platform.<sup>16</sup> Functional analysis was restricted to samples with at least 5,000,000 reads after quality filtering and *Homo sapiens* read removal, in order to ensure adequate sequencing depth and comparability across samples. Reads were functionally classified to infer metabolic pathways based on the Rhea database (<https://www.rhea-db.org>)<sup>16</sup> using RAPSearch2 software,<sup>55,56</sup> following standard METAnnotatorX2 parameters, including an e-value threshold of  $1 \times 10^{-8}$  and a minimum query coverage of 80%.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted using SPSS (v25) and R (v4.1.2) to examine the relationship between FC levels, confounding factors (sex, age, body mass index, chronic diseases, anxiety/depressive disorders, previous infections, intestinal habits, smoking and alcohol consumption, drug use, and nutritional supplements), and microbiota composition. Initially, the Mann–Whitney U test was employed to assess potential differences in FC levels based on sex and age groups, i.e., G3 (18–64 years) and G4 (65 years and older), then Fisher's Exact Test was applied to account for confounding factors.

Microbial beta-diversity differences between groups with normal and high FC levels were analyzed using PERMANOVA based on the Bray–Curtis dissimilarity index, implemented in QIIME2. ADONIS analysis was performed to assess the effects of specific factors like alcohol consumption and chronic use of proton pump inhibitors on microbial composition. For intra-individual bacterial diversity, alpha-diversity metrics (species richness and Shannon indices) were calculated, and differences between the normal and high FC groups were tested using the Mann–Whitney U test.

Taxonomic and functional differences between the two groups were evaluated through a combination of methods, including relative difference in abundance, the Mann–Whitney U test, Partial Least Squares Discriminant Analysis (PLS-DA), and RG (Group Indicators) analysis. In detail, the PLS-DA analysis was conducted using custom R scripts that used the *plsda* and *vip* functions to extract Variable Importance in Projection (VIP) scores. Moreover, RG analysis was performed using the *multipatt* function from the *indicspecies* R package. The *r.g* statistic for each taxon was computed, and taxa were selected based on permutation testing (999 iterations) with a significance threshold of *p*-value <0.05.

To investigate the relationship between FC levels and microbiota composition or functionality, Spearman correlation analysis was conducted in R using the *Hmisc* package. This analysis examined correlations between FC levels, total cell counts, and the relative abundance of bacterial taxa, adjusting *p*-values for multiple testing using the Benjamini–Hochberg (FDR) corrections.