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Children's Inflammatory Bowel Disease and Methanogenic Archaea: Links to Disease Classification and Activity

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Abstract

Inflammatory bowel disease (IBD) involves disruptions in the gut microbial ecosystem, yet research on methanogenic archaea in children is scarce. This study investigated the presence and abundance of total methanogenic archaea and three specific groups—Methanobrevibacter smithii (Mb. smithii), Methanosphaera stadtmanae (Ms. stadtmanae), and the Methanomassiliicoccales order—in the fecal samples of pediatric patients with Crohn's disease (CD) and ulcerative colitis (UC), considering both active and inactive disease states. Quantitative real-time PCR data were analyzed in relation to disease type and activity, assessed via the Pediatric Crohn's Disease Activity Index (PCDAI), Pediatric Ulcerative Colitis Activity Index (PUCAI), and fecal calprotectin (FCP), and compared with healthy children. Total methanogen counts were significantly lower in both CD and UC patients than in controls, with UC showing the lowest prevalence. In inactive UC, Mb. smithii levels were reduced, and Ms. stadtmanae abundance displayed a moderate positive correlation with FCP concentrations. These findings suggest that methanogenic archaea profiles vary according to IBD type and activity in pediatric patients.

Keywords: Children, Inflammatory bowel disease, Crohn's disease, Ulcerative colitis, Methanogens, Archaea

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Introduction

IBD, comprising Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disorder of the gastrointestinal tract characterized by recurrent diarrhea, abdominal pain, appetite loss, and weight reduction [1]. Severe cases may lead to complications such as ulcers, fistulas, bowel obstruction, or sepsis [1]. In developed countries, IBD affects up to 0.3% of the population [2], and approximately 25% of new cases are diagnosed in individuals under 18 years of age [3]. The highest rates are observed in Western Europe (40–50 per 100,000 per year) and North America (3.1–14.6 per 100,000 per year) [4]. While IBD can occur at any age, onset commonly peaks in the second and third decades, with 15% of cases involving children [5]. In Poland, the pediatric incidence is 2.8 per 100,000 per year in children under 15 [3]. Its etiology is multifactorial, involving genetic susceptibility, immune dysregulation, microbial imbalances, and environmental factors such as diet and residence [6, 7].

Intestinal dysbiosis is recognized as a key contributor to IBD development [8], and emerging evidence points to changes in methanogenic archaea as well [9]. Previously considered extremophiles [10], archaea were first identified in the human gut in 1968 with the isolation of methanogens [11], establishing them as normal intestinal inhabitants [12]. In healthy individuals, methanogens can constitute up to 10% of gut anaerobes [13], with Mb.

smithii, Ms. stadtmanae, and the Methanomassiliicoccales order being the most prominent [14]. Their effects on the host differ: Mb. smithii is largely commensal [15], whereas Ms. stadtmanae provokes strong immune responses in both healthy individuals and IBD patients [13, 16, 17].

Adult studies show reduced total methanogens and lower Mb. smithii levels in IBD, alongside elevated proportions of Ms. stadtmanae [9, 16]. Such patterns remain unconfirmed in children [18, 19]. Pediatric IBD differs from adult-onset disease in progression, anatomical involvement, and treatment response [20], making it essential to study children separately. Moreover, children offer a clearer model for investigating IBD mechanisms due to minimal comorbidities [8]. Accordingly, this study examined fecal methanogenic archaea in children with CD and UC, and explored associations with disease type and activity relative to healthy controls.

Results and Discussion

Study population

Ninety-seven children with IBD were enrolled, including 45 with CD (mean age 14.2 years) and 52 with UC (mean age 13.0 years) **(Table 1)**. Participants were grouped based on disease activity (active vs. inactive), determined using fecal calprotectin levels and disease-specific indices (PCDAI for CD and PUCAI for UC). No significant differences were observed in age or sex between CD and UC groups. Mean PCDAI and PUCAI scores were 10.0 (±13.5) and 15.2 (±20.3), respectively, and were significantly higher in active versus inactive disease ($p = 0.0027$ and $p = 0.0017$). FCP concentrations averaged 781.6 (±1800.7) µg/g in CD and 396.6 (±810.3) µg/g in UC, with significantly higher levels in active cases compared to inactive cases ($p < 0.0001$ and $p = 0.0002$) and controls (19.3 ± 24.1 µg/g; $p < 0.0001$).

Table 1. Characteristics of the study participants

Group	Female:Male	Age [Years] Mean ± SD (Median)	FCP [µg/g] Mean ± SD (Median)	PUCAI Mean ± SD (Median)	PCDAI Mean ± SD (Median)
CD (n = 45)	25:20	14.2 ± 3.1 (15.0)*	781.6 ± 1800.7 (119.0)*	n/a	10.0 ± 13.5 (5.0)
Inactive CD (n = 24)	10:14	14.6 ± 2.8 (15.0)*	64.0 ± 61.0 (59.5)	n/a	3.4 ± 7.8 (0.0)
Active CD (n = 21)	15:6	13.9 ± 3.4 (15.0)*	1601.8 ± 2409.4 (699.0)*#	n/a	17.5 ± 14.7 (15.0)#
UC (n = 52)	27:25	13.0 ± 4.7 (14.0)*	396.9 ± 810.3 (100.5)*	15.2 ± 20.3 (5.0)	n/a
Inactive UC (n = 27)	15:12	13.8 ± 4.3 (15.0)*	62.0 ± 54.9 (38.0)	3.7 ± 4.9 (0.0)	n/a
Active UC (n = 25)	12:13	12.2 ± 5.0 (13.0)	758.5 ± 1062.9 (403.0)*#	27.6 ± 23.3 (25.0)#	n/a
Controls (n = 27)	14:13	10.0 ± 4.0 (10.0)	19.3 ± 24.1 (10.0)	n/a	n/a
Inactive UC (n = 27)	15:12	13.8 ± 4.3 (15.0)*	62.0 ± 54.9 (38.0)	3.7 ± 4.9 (0.0)	n/a
Active UC (n = 25)	12:13	12.2 ± 5.0 (13.0)	758.5 ± 1062.9 (403.0)*#	27.6 ± 23.3 (25.0)#	n/a
Controls (n = 27)	14:13	10.0 ± 4.0 (10.0)	19.3 ± 24.1 (10.0)	n/a	n/a

Notes

n/a: not applicable; SD: standard deviation; Median values are shown in parentheses; PCDAI: Pediatric Crohn's Disease Activity Index; PUCAI: Pediatric Ulcerative Colitis Activity Index; FCP: fecal calprotectin; CD: Crohn's disease; UC: ulcerative colitis.

* $p < 0.05$ versus controls; # $p < 0.05$ versus inactive group of the same disease type. Statistical comparisons were performed using the Kruskal–Wallis H test.

No significant differences in age were observed between children with active UC and the control group ($p > 0.05$).

Prevalence and levels of gut methanogens

Initially, both qualitative and quantitative assessments of methanogens were conducted on the fecal samples from all participants. Unlike the control group, in which methanogens were detected in every child, a notable proportion of children with IBD showed no detectable methanogens **(Table 2)**. The overall prevalence of methanogens was significantly reduced in children with UC and those with active UC ($p < 0.05$). Further examination of specific methanogen groups revealed that Ms. stadtmanae was detected significantly less frequently in UC patients compared to controls ($p < 0.05$). Additionally, the likelihood of identifying each methanogen subgroup was generally lower across most IBD groups—except for children with active CD—relative to healthy controls **(Table 3)**.

Table 2. Prevalence of methanogens [%] in pediatric groups with active and inactive IBD and in healthy controls

Group	Total Methanogens	*Ms. stadtmanae*	*Mb. smithii*	Methanomassiliicoccales
CD (n = 45)	40/45 [88.9%]	12/45 [26.7%]	31/45 [68.9%]	6/45 [13.3%]
Inactive CD (n = 24)	21/24 [87.5%]	4/24 [16.7%]	15/24 [62.5%]	3/24 [12.5%]

Active CD (n = 21)	19/21 [90.5%]	8/21 [38.1%]	16/21 [76.2%]	3/21 [14.3%]
UC (n = 52)	43/52 [82.7%]*	8/52 [15.4%]*	27/52 [51.9%]	8/52 [15.4%]
Inactive UC (n = 27)	23/27 [85.2%]	4/27 [14.8%]	12/27 [44.4%]	6/27 [22.2%]
Active UC (n = 25)	20/25 [80.0%]*	4/25 [16.0%]	15/25 [60.0%]	2/25 [8.0%]
Controls (n = 27)	27/27 [100%]	10/27 [37.0%]	20/27 [74.1%]	8/27 [29.6%]

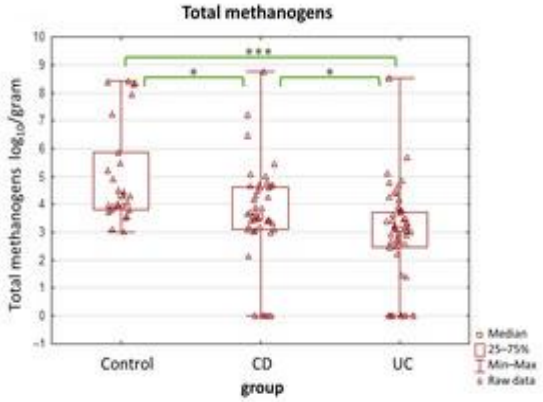
The findings show the proportion of children with stool samples positive for total methanogens, Mb. smithii, Ms. stadtmanae, and Methanomassiliicoccales. The presence of methanogenic archaea was determined using real-time PCR [21]. * indicates statistical significance ($p < 0.05$) relative to the control group. CD refers to Crohn's disease, and UC refers to ulcerative colitis. Statistical comparisons were conducted using Fisher's exact test.

Table 3. Odds ratios for the detection of methanogens in pediatric patients with active and inactive IBD relative to control subjects

Group	Total Methanogens *	Ms. stadtmanae	Mb. smithii	Methanomassiliicoccales
CD (n = 45)	0	0.62 [0.22, 1.72]	0.78 [0.27, 2.25]	0.37 [0.11, 1.20]
Inactive CD (n = 24)	0	0.34 [0.09, 1.28]	0.58 [0.18, 1.92]	0.34 [0.08, 1.47]
Active CD (n = 21)	0	1.05 [0.32, 3.40]	1.12 [0.30, 4.20]	0.40 [0.09, 1.73]
UC (n = 52)	0	0.31 [0.10, 0.91]	0.38 [0.14, 1.05]	0.43 [0.14, 1.32]
Inactive UC (n = 27)	0	0.30 [0.08, 1.11]	0.28 [0.09, 0.88]	0.68 [0.20, 2.31]
Active UC (n = 25)	0	0.32 [0.09, 1.22]	0.53 [0.16, 1.70]	0.21 [0.04, 1.09]

* No control subjects were negative for total methanogens, resulting in a zero value. CD—Crohn's disease; UC—ulcerative colitis. Odds ratios were calculated using the OpenEpi web tool [22].

In children with CD, UC, and healthy controls, the mean total methanogen abundance was 3.66, 2.87, and 5.04 $\log_{10}$/g of dry weight, respectively, while the corresponding median values were 3.55, 3.07, and 4.30 $\log_{10}$/g. Analysis revealed a statistically significant reduction in total methanogenic archaea in both CD and UC groups compared to controls ($p < 0.05$) **(Figure 1a)**. Additionally, total methanogen levels differed significantly between CD and UC patients. When the individual methanogen subgroups—Mb. smithii, Ms. stadtmanae, and Methanomassiliicoccales—were analyzed separately, only Mb. smithii exhibited a significant decrease in UC patients relative to controls ($p = 0.0015$) **(Figure 1b)**. No significant differences were detected for Ms. stadtmanae or Methanomassiliicoccales **(Figures 1c and 1d)**.



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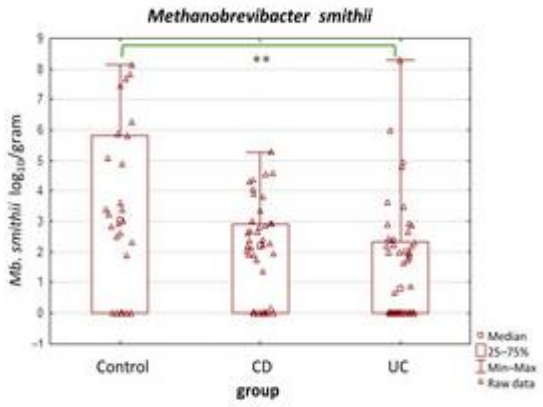

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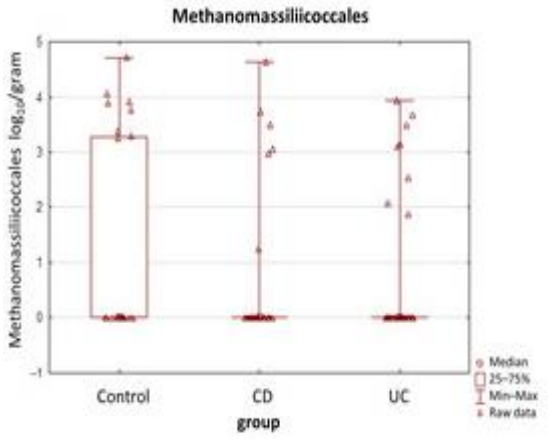

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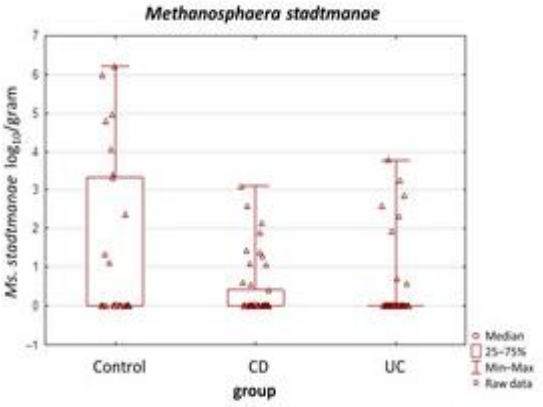
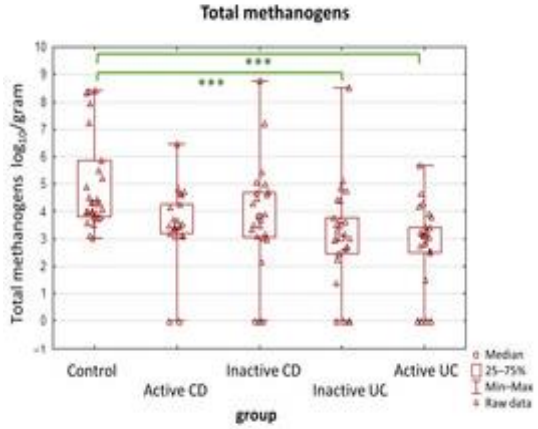


d)

Figure 1. (a–d) Comparison of methanogen abundance (per gram of dry stool) among children with Crohn's disease (CD), ulcerative colitis (UC), and healthy controls. The most marked differences were observed in the total methanogen population (a). Significance levels were indicated as *p < 0.05, **p < 0.01, and ***p < 0.001; non-significant differences were left unmarked. Statistical analysis was conducted using the Kruskal–Wallis H test.

Further stratification based on disease activity in the IBD groups produced similar patterns for UC versus controls. Both active and inactive UC patients exhibited markedly lower total methanogen counts compared to controls ($p = 0.0002$ and $p < 0.0001$, respectively) **(Figure 2a)**. In contrast, CD patients showed no statistically significant differences in total methanogen abundance between either active or inactive subgroups and the control group. Regarding Mb. smithii, a significant reduction was observed only in the inactive UC group compared to controls ($p = 0.002$) **(Figure 2b)**. The abundances of Methanomassiliicoccales and Ms. stadtmanae did not show significant differences in any comparison **(Figures 2c and 2d)**.



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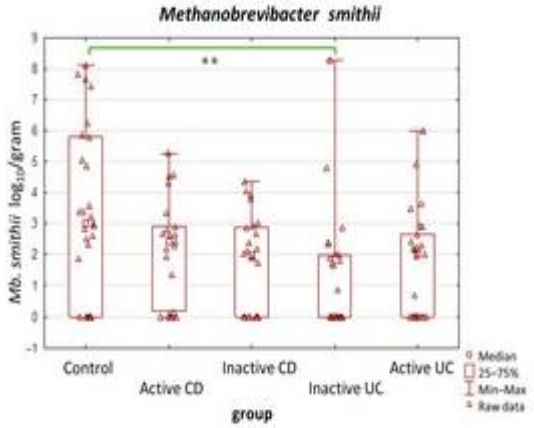

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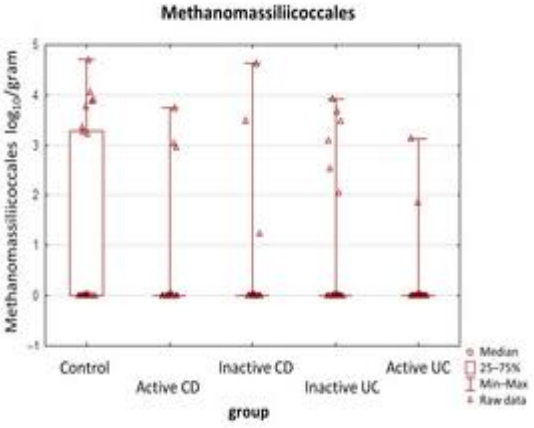

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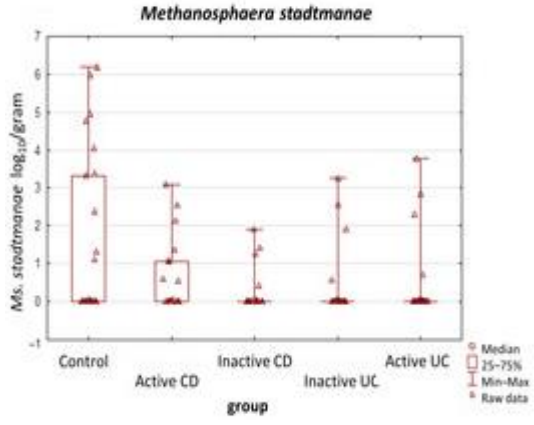

d)

Figure 2. (a–d) Comparison of methanogen abundances (per gram of dry stool) among children with active and inactive Crohn's disease (CD), active and inactive ulcerative colitis (UC), and healthy controls. The largest differences were observed for the total methanogen population (A). Significance was indicated as **p < 0.01 and ***p < 0.001; non-significant differences were left unmarked. Statistical comparisons were performed using the Kruskal–Wallis H test.

Association between IBD activity scores and methanogenic archaea abundance

Correlation analyses between disease activity scores—PCDAI for CD and PUCAI for UC—and methanogen abundance showed a single moderate positive association between total methanogen levels and PCDAI in patients with active CD (Rs = 0.48, p = 0.026) **(Figure 3)**. No significant correlations were observed between disease activity and the abundance of the other archaeal subgroups.

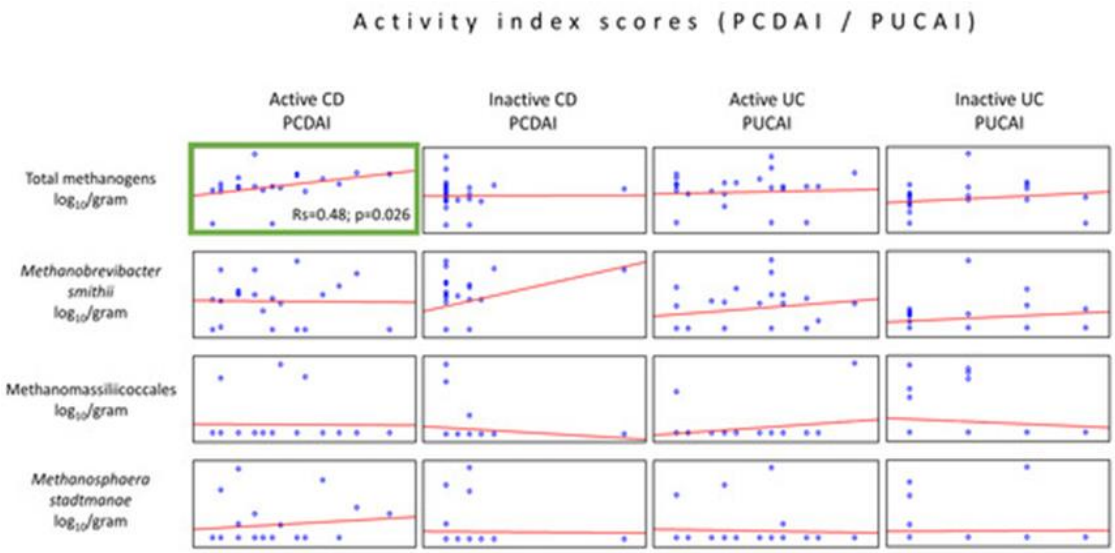

Figure 3. Correlations between the quantities of methanogens and the disease activity indices (PCDAI or PUCAI) in stool samples (dry weight) from patients in the IBD groups. Statistically significant correlations were found only for the total methanogen population, which showed an increase corresponding to higher PCDAI scores (highlighted in the green box).

Association between FCP and methanogenic archaea abundance

Since FCP levels are known to reflect IBD severity, we examined the relationship between FCP concentrations and the abundance of total methanogens, Mb. smithii, Ms. stadtmanae, and Methanomassillicoccales. Analysis within individual disease activity groups identified a single moderately positive correlation between Ms. stadtmanae abundance and patients with inactive UC (Rs = 0.41, p = 0.034) **(Figure 4)**.

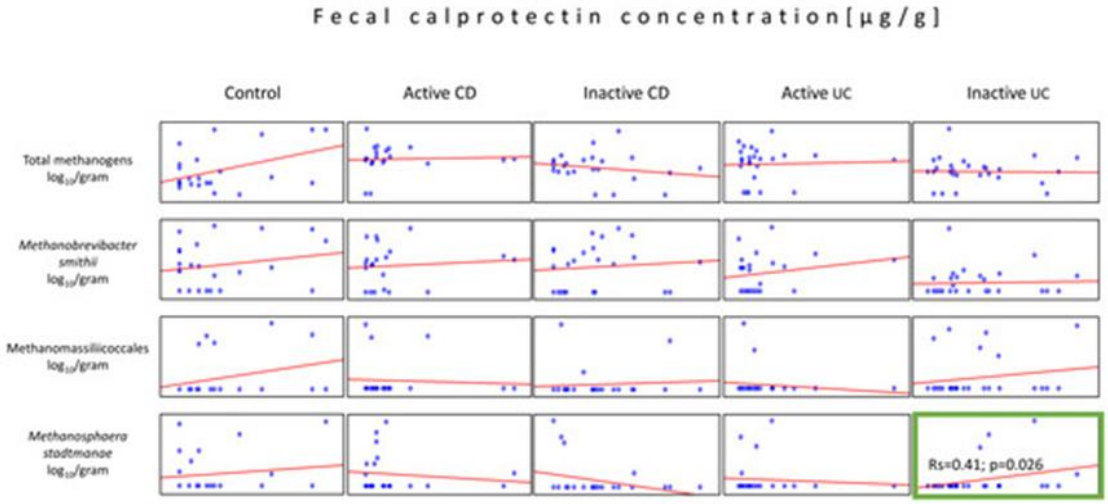

Figure 4. Relationship between methanogen levels and fecal calprotectin (FCP) concentrations in dry stool samples across all study groups. A statistically significant correlation with FCP was found exclusively for Ms. stadtmanae in the inactive UC cohort (green box).

Influence of age on methanogenic archaea abundance

Because the control children and those with IBD differed considerably in age **(Table 1)**, we assessed how age might relate to methanogen counts. The mean ages were 13.9 ± 3.4 years for active CD, 14.6 ± 2.8 years for inactive CD, 12.2 ± 5.0 years for active UC, 13.8 ± 4.3 years for inactive UC, and 10.0 ± 4.0 years for controls. Among the

IBD patients, only active CD showed a significant age-related trend: total methanogen numbers and Mb. smithii abundance declined with increasing age (Rs = –0.56, p = 0.009; Rs = –0.53, p = 0.013). In contrast, in the control group, Methanomassiliicoccales counts rose significantly as age increased (Rs = 0.49, p = 0.009) **(Figure 5)**.

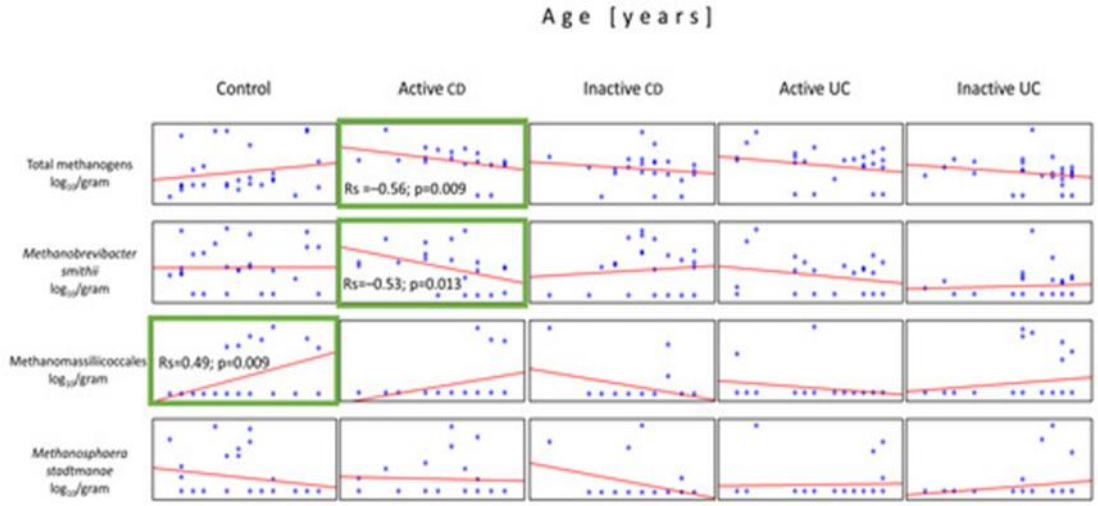

Figure 5. Association between methanogen abundances and patient age in dry stool samples across all groups. Statistically significant age-related correlations were detected only for Methanomassiliicoccales in controls and for total methanogens and Mb. smithii in active CD patients (highlighted in green boxes).

Alterations in the intestinal microbiome appear to contribute substantially to IBD pathogenesis, yet research specifically addressing the role of methanogens is limited, and most available studies focus on adults [9, 16, 23–25]. To date, detailed analyses of intestinal methanogens in pediatric CD have been reported by only a single group [19]. To our knowledge, this study is the first to examine methanogen prevalence in UC independently from CD and to explore relationships between four distinct methanogen groups and disease activity in both pediatric IBD types.

Our findings revealed a marked reduction in total methanogen abundance in both CD and UC compared with controls, representing the sole shared characteristic between these two diseases, which is unsurprising given their distinct pathophysiology and disease courses [3]. In UC patients, methanogens were not only less abundant but also less frequently present than in controls, particularly for lower-level taxa such as Mb. smithii. This supports previous observations that reduced Mb. smithii is a consistent indicator of gut dysbiosis in IBD [23, 26, 27].

Regarding Ms. stadtmanae, prior studies in adults suggest it can trigger strong inflammatory responses, even in healthy intestines, more so than other methanogens [16, 28]. Although our data did not confirm this directly, we observed a noteworthy finding consistent with this notion: a significant positive correlation between Ms. stadtmanae levels and fecal calprotectin (FCP) in inactive UC patients. FCP is a well-established marker of intestinal inflammation and IBD severity [29]. Interestingly, a similar, though statistically nonsignificant, trend was seen across all four methanogen groups in control children, suggesting that multiple intestinal methanogens may contribute variably to mucosal inflammatory processes.

In contrast, pediatric CD patients exhibited methanogen prevalence similar to that of controls across all four groups, aligning partially with findings reported by Krawczyk *et al*., (2021) [19]. However, prevalence rates differed: in our study, Mb. smithii was detected in 76% of active CD, 63% of inactive CD, and 74% of controls, compared to 44%, 27%, and 36%, respectively, in the previous study. For Ms. stadtmanae, our percentages were 38%, 17%, and 37% in active CD, inactive CD, and controls, whereas the earlier study reported around 30% for all groups [19]. Notably, unlike in adult IBD populations where Ms. stadtmanae is approximately three times more prevalent, only 27% of CD and 15% of UC children were colonized, compared with 37% of controls [16].

Several adult IBD findings are partially reflected in our pediatric data, highlighting the value of comparing child and adult results in future research. In adults, reduced methane production in IBD is well-documented [30–33]. Considering that at least 8 log10/g dry feces is required to detect methane in breath [34], most children in the active IBD groups would likely be non-methane producers. In contrast, some children in the control group could produce methane—specifically, 2 of 15 (13%) aged 8–14 and 2 of 5 (40%) aged 14–18—which aligns with Peled *et al*., (1985), who reported 14–18% and 9–46% methane producers in these age ranges [35].

Because the control group was age-matched only with active UC patients, we examined age-related trends in methanogen abundance across all IBD subgroups.

Interestingly, active CD patients showed a significant age-dependent decrease in total methanogens and Mb. smithii. Similar declining trends were observed in other IBD groups, though not statistically confirmed. Furthermore, a positive correlation emerged between PCDAI scores and total methanogen counts in active CD patients. Whether this pattern reflects a causal role of methanogens in disease onset or a consequence of disease progression remains unclear; however, it is tempting to speculate that methanogens may contribute to disease initiation (as suggested by the PCDAI–methanogen correlation), with their abundance diminishing over time as the disease persists, particularly in its active form. This hypothesis warrants further investigation [19].

Regarding age-related variations, an additional observation emerged: in the control group, Methanomassiliicoccales showed a marked increase as children grew older. This pattern, previously documented in both adults and children [36, 37], is further supported by our findings. Additionally, Vanderhaeghen *et al*. (2015) reported that Methanomassiliicoccales can occasionally outnumber Methanobacteriales (mainly Mb. smithii) [38], a trend confirmed in our study and observed across multiple patient groups rather than being limited to isolated cases.

Limitations and strengths of the study

The findings should be interpreted with caution due to several limitations. The study involved a relatively small cohort, limiting statistical power. The control group was significantly younger than patients with active and inactive CD and inactive UC, which could have influenced some outcomes. The potential effects of medications cannot be excluded, as patients received diverse treatments grouped into five categories: 5-ASA agents (mesalazine), immunosuppressants (azathioprine), biological drugs (infliximab, adalimumab, vedolizumab, ustekinumab), steroids (prednisone, budesonide), and nutritional therapy. Although literature suggests that certain IBD treatments may influence methanogen abundance—mesalazine, for example, has been reported to increase or decrease specific methanogens [16, 23, 27]—no such association was observed in our study, likely due to the limited number of patients per therapy, which may have obscured potential correlations. Importantly, methanogen-negative samples were not confined to any single drug group, indicating that no specific therapy completely eliminated methanogens. Notably, low Mb. smithii levels have been proposed as a marker for predicting response to anti-TNF therapy in IBD [39], a topic warranting further research. It is also possible that pediatric patients harbored other unexamined microbial taxa that might influence IBD development, as the most significant differences between patient groups were seen in total methanogen counts rather than individual methanogen subgroups, leaving questions about methanogenic diversity in children's intestines unresolved.

Conversely, this study is, to our knowledge, the first to separately assess the prevalence and abundance of four methanogen groups in pediatric UC and CD and to correlate methanogen levels with FCP and PUCAI in UC.

Materials and Methods

Subjects

A total of 124 children participated in the study, including 97 IBD patients and 27 controls. All patients were treated at the Department of Gastroenterology, Hepatology, Nutritional Disorders and Pediatrics, Children's Memorial Health Institute (Warsaw, Poland), with disease activity assessed according to European Crohn's and Colitis Organization (ECCO) guidelines [40]. Participants ranged from 3 to 18 years old, comprising 45 CD patients and 52 UC patients. The control group included 27 children referred for fecal testing for non-IBD conditions. IBD patients were further classified into active and inactive CD (21 and 24 patients, respectively) and active and inactive UC (25 and 27 patients, respectively).

Subgroup classification was based on PCDAI or PUCAI scores and fecal calprotectin (FCP) levels. CD was considered inactive with PCDAI <12.5 and active with PCDAI >20; three children with scores between 12.5 and 20 were included in the active CD group based on disease progression. UC patients with PUCAI <10 were considered in remission, scores between 10 and 30 indicated mild UC, and scores above 30 indicated active exacerbation; thus, "inactive UC" encompassed both remission and mild cases. Patients with low activity scores but elevated FCP (>200 μg/g) were classified into the active IBD groups.

FCP measurement

Fecal calprotectin (FCP) concentrations were determined using magnetic microparticle chemiluminescence on the Liaison® XL platform (DiaSorin, Saluggia, Italy). The assay relies on a dual-antibody system: one mouse monoclonal antibody attached to particles captures FCP from the stool, while a second conjugated monoclonal antibody, recognizing a different FCP region, enables detection. Approximately 15 mg of freshly collected feces was transferred into extraction buffer using a serrated stick. The method's measurable range was 5–800 μg FCP per gram of stool, and samples exceeding this range were diluted, with results multiplied by the corresponding dilution factor.

DNA extraction

DNA was extracted from 100 mg of each stool sample placed in 2 mL microtubes; samples with higher water

content were increased up to 300 mg, and adjustments were applied to the final calculations. The extraction protocol was based on a previously described procedure [41] but incorporated an improved mechanical lysis step using bead-beating instead of sonication to reduce handling time. Briefly, fecal material was homogenized in BS buffer (A&A Biotechnology, Gdynia, Poland) until a viscous suspension formed. Lysozyme (30 µL, 10 mg/mL) and mutanolysin (7 µL, 10 U/µL) were added, followed by incubation at 37 °C for 15 minutes and then at 50 °C for 25 minutes. Subsequently, 700 µL LS lysis buffer and 35 µL proteinase K (20 mg/µL) were added, with a 1-hour incubation at 50 °C. After centrifugation at 14,000 rpm for 5 minutes, the supernatant was collected, and the remaining debris was mixed with zirconia/silica beads and 500 µL LS buffer for bead-beating in a TissueLyser LT (Qiagen, Hilden, Germany) at 50 oscillations per second for 3 minutes, following Salonen *et al*., (2010) [42]. The samples were centrifuged again, the bead-beating step repeated, and the final lysate incubated at 95 °C for 5 minutes to enhance cell lysis. The resulting three supernatant fractions were combined and purified using the Genomic Mini AX Bacteria+ kit (A&A Biotechnology) according to the manufacturer's protocol [41].

Quantitative real-time PCR

Quantitative PCR was carried out following the protocol described previously [21]. The targets included the mcrA gene (methyl-coenzyme M reductase α-subunit) for total methanogenic archaea, the nifH gene (non-functional nitrogenase) for Mb. smithii, 16S rRNA for Methanomassiliicoccales, and the mtaB gene (coenzyme M methyltransferase) for Ms. stadtmanae. Primer specificity **(Table 4)** was experimentally validated and confirmed with BLAST, and the copy number of each amplicon per organism was verified using the Ribosomal RNA Database [43]. All genes were single-copy per genome except for mtaB; for Ms. stadtmanae, cell counts were corrected by dividing PCR results by four, reflecting the number of operons in strain DSM 3091 (CP000102.1). Standard curves were generated using serial decimal dilutions ranging from approximately 100 to 10^6 copies per reaction, prepared from genomic reference DNAs: a linearized plasmid containing an mcrA fragment (KF214818.1:976–1447) and purified amplicons of nifH, 16S rDNA, and mtaB obtained from human fecal samples aligning with Methanobrevibacter smithii KB11 (CP017803.1), uncultured Methanomassiliicoccus sp. (LC473299.1), and Methanosphaera stadtmanae MGYG-HGUT-02164 (LR698975.1), respectively. DNA concentrations of the standards were quantified using a Quantus fluorometer with the QuantiFluor dsDNA System (Promega, Madison, WI, USA) and converted to genome copies per microliter using the Science Primer web tool [44].

Table 4. Primers utilized for the detection of total methanogens and their three subgroups

Microorganism	Reference	Target Gene	Primer Sequence 5′–3′ (Forward / Reverse) *	Amplicon Size [bp]
Total methanogenic archaea	[21]	mcrA	CTTGAARMTCACTTCGGTGGWTC / CGTTCATBGCGTAGTTVGGRTAGT	~270
Methanobrevibacter smithii	Modified from [45]	nifH	AACAGAAAACCCAGTGAAGAGGATA / ACGTAAAGGCACTGAAAAACCTCC	222
Methanomassiliicoccales	Modified from [46]	16S rDNA	GGGGTAGGGGTAAAATCCTGTAATCC / AACAACTTCTCTCCGGCACTGG	194
Methanosphaera stadtmanae	Modified from [16]	mtaB	GTAGTTCCTAACATCAAAGTAGCTCC / TCCTCTAAGACCGTTTTCTTCTTCTCTCA	300

* The original oligonucleotide sequences from the cited sources are indicated with underlining

Table 5. Temperature settings used for the absolute quantification of each group of methanogenic archaea.

Real-Time PCR Step	Total Methanogens	Mb. smithii	Methanomassiliicoccales	Ms. stadtmanae
Initial Denaturation	95 °C—5 min			
Denaturation	94 °C—20 s	94 °C—20 s	94 °C—20 s	94 °C—20 s
Annealing	60 °C—20 s	66 °C—20 s	70 °C—20 s	68 °C—20 s
Elongation	72 °C—20 s	72 °C—20 s	72 °C—20 s	72 °C—25 s

Signal acquisition *	81 °C—20 s + Acq	82 °C—20 s + Acq	87 °C—20 s + Acq	81 °C—20 s + Acq
Melt analysis *	95 °C—5 s, then 60 °C—1 min, and 95 °C—continuous Acq with ramp rate 0.11 °C/s			

* Acq—acquisition of fluorescence signal.

Statistical analysis

All statistical analyses were conducted using TIBCO Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Normality of the quantification data was evaluated with the Shapiro–Wilk test, and variance homogeneity was assessed using Levene's test. Differences among groups were analyzed using the non-parametric Kruskal–Wallis H test to examine (1) variation in methanogen abundances according to disease type and activity, and (2) associations of IBD groups with age, PCDAI or PUCAI scores, and fecal calprotectin (FCP) levels. Spearman's rank correlation was applied to determine the strength and direction of associations between methanogen levels, disease activity, calprotectin, and age. Following the interpretation criteria of Prion and Haerling (2014) [47], correlation coefficients (Rs) were classified as very strong (0.81–1), strong (0.61–0.80), moderate (0.41–0.60), weak (0.21–0.40), or negligible (0–0.20). Methanogen prevalence was evaluated using Fisher's exact test, and odds ratios were calculated via two-by-two tables in the OpenEpi web tool [22].

Conclusion

The study demonstrated that gut methanogenic archaea are linked to pediatric IBD type, with the most pronounced effects observed in children with UC, where reduced methanogen levels were associated with disease presence. Notably, this is the first study to suggest a potential role for Ms. stadtmanae in UC, as its abundance in the inactive UC group showed a positive correlation with elevated FCP, a biomarker of mucosal inflammation. Although these findings are promising, larger-scale studies are required to clarify the role of methanogenic archaea in the pathogenesis of pediatric IBD.

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Conflict of interest: None

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Ethics statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee from the Children's Memorial Health Institute (protocol code 49/KBE/2019, approval date 6 November 2019).

Informed consent was obtained from participants over 16 years of age and/or their legal representative, as appropriate.

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