Int J Colorectal Dis (2012) 27:171–178
DOI 10.1007/s00384-011-1323-4

ORIGINAL ARTICLE

Colonic methanogenesis in vivo and in vitro and fecal pH after resection of colorectal cancer and in healthy intact colon

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Accepted: 4 October 2011 / Published online: 18 October 2011

Abstract
Purpose We compared colonic methanogenesis in vivo and in vitro as well as fecal pH in healthy subjects and in patients with resected colorectal cancer thus without the possible confounding effects of the tumor.
Methods A total of 144 subjects, 96 with resected colorectal cancer (of whom, 48 were with metastatic disease), 48 healthy subjects with intact colon, were analyzed for breath methane, fecal methanogenesis in vitro and fecal pH. In addition, the association between methanogenesis and pH with cancer site, operation technique and abdominal discomfort was investigated.
Results In vivo and in vitro methane measurements were in agreement. The percentage of breath methane excretors and fecal pH did not significantly differ in participants resected for colorectal cancer, either with (46%, 6.76) or without (46%, 6.77) metastatic disease, from healthy participants (40%, 6.80). Breath methane excretors had higher fecal pH than nonexcretors (7.05 versus 6.57, $P<0.001$) and less abdominal discomfort (30% versus 54%, $P=0.016$). Among patients with resected right-sided cancer ($n=15$), there were less breath methane excretors (20%) than among those with resected left-sided cancer (51%, $n=81$, $P=0.029$) as well as lower fecal pH than among those with resected left-sided cancer (6.27 versus 6.86, $P=0.002$) and among healthy subjects (6.80, $P=0.010$).
Conclusions Patients with resected colorectal cancer were as frequently methane producers as healthy subjects with intact colon, and there was no difference in their fecal pH. Low methanogenesis was found in patients with abdominal discomfort and is a possible characteristic, along with low fecal pH, to right-sided colorectal cancer.

Keywords Abdominal discomfort · Colorectal cancer · Fecal pH · Intestinal microbiota · Methane

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Introduction

Intestinal microbiota is probably a major environmental modulator of colonic cancer risk in humans [1, 2]. Intestinal microbiota is composed of over a thousand distinct bacterial species or phylotypes but extremely reduced diversity of archea [3]. In contrast to most of the metabolic

groups of microorganisms in the colon of healthy humans, significant interindividual differences have been found in methanogenic archaea [4], namely *Methanobrevibacter smithii* and the less frequently found *Methanosphaera stadtmanae*, which use H_2 to produce methane (CH_4) [3, 5–7]. Together with sulfate-reducing and reductive acetogenic bacteria, methanogens transfer H_2 to other species and regulate the activity of the overall microbiota [3]. Methanogens could, therefore, theoretically influence human health by supporting the growth of fermenting bacteria, either commensals or pathogens [3]. They also have a high potential to transform heavy metals into more toxic volatile methylated derivatives, such as trimethylbismuth [$(CH_3)_3Bi$] and dimethylselenium [$(CH_3)_2Se$] [3, 8, 9]. Some large studies have detected low colonic methanogenesis in groups with high risk of colon cancer and high colonic methanogenesis in groups with low risk [10, 11]. In contrast, some other studies have found that patients with colon cancer are more likely to be CH_4 producers than subjects with nonmalignant colon disorders or without colon disorders [12, 13], leaving the possible role of CH_4 in colorectal cancer controversial.

Methanogens, as well as the majority of harmful bacterial enzymes, operate optimally at neutral to slightly basic pH [6, 14]. Fecal pH is mainly determined by the balance between the production and absorption of short-chain fatty acids (SCFA) and ammonia, which are produced in the colon by bacterial fermentation of carbohydrates and proteins, respectively [15]. SCFA, especially butyrate, are considered potentially anticarcinogenic [16]. Ammonia, on the other hand, may be involved in tumor promotion [17]. The presence of a connection between colonic pH and colorectal cancer has not been convincingly shown. However, some studies have detected a higher fecal pH in patients with colorectal cancer (mainly distal disease) or with resected sigmoid colon cancer when compared to healthy subjects [18, 19].

Approximately half of the CH_4 produced is absorbed and excreted in expired air [7]. Since CH_4 is neither produced nor used by host cells or other colonic organisms, breath CH_4 excretion can be used as an indicator of the in situ activity of the methanogenic microbiota [6, 7]. Indeed, it is the reference method for measuring colonic CH_4 production. We also wanted to explore colonic methanogenesis directly by fecal fermentation in vitro, since several subjects have been reported to excrete no CH_4 in the breath despite CH_4 being present in colonic gas [10].

The main objective of this study was to investigate methanogenesis and pH in the colon of patients that have undergone bowel resection due to colorectal cancer and in healthy volunteers with intact colon. We included colorectal cancers operated by conventional surgical techniques with partial resection of the large bowel. Because the time from operation to adjuvant chemotherapy has to be short, the effect of the operation could not be excluded (in radically resected patients). Thus, a second patient group with a longer time interval from operation to sampling and a more advanced stage of the disease was included (the metastatic patients). The secondary objectives were to evaluate the connections between methanogenesis and fecal pH with cancer site, operation technique, as well as abdominal discomfort. To the best of our knowledge, such connections have not been investigated before.

Patients and methods

Study design

A total of 144 subjects, of whom 96 had resected colorectal cancer, participated in the study. We included colorectal cancer patients operated by conventional surgical techniques with partial resection of the large bowel. Colorectal cancer patients with gastrointestinal diseases, such as colitis, gluten intolerance, previous debilitating gastrointestinal operations or symptomatic carcinomatosis, were excluded from the study.

Three study groups were formed as follows. The radically resected cancer group consisted of 48 consecutive colorectal cancer patients with histologically confirmed colorectal stage II to III tumor that had been radically removed at surgery, without metastases in radiological examinations, and who were referred to the Helsinki University Central Hospital, Department of Oncology, for adjuvant chemotherapy after resection. Age- and gender-matched pairs to these patients were recruited into the two other groups. The metastatic cancer group (n=48) consisted of patients referred to the Department of Oncology for treatment of colorectal carcinoma who had a history of cancer resection and had been diagnosed as having metastatic colorectal cancer with adenocarcinoma histology. The healthy subjects (n=48) were recruited among Valio Ltd. (Helsinki, Finland) employees and other healthy volunteers with no history of gastrointestinal disease and without bowel symptoms (checked by a structured questionnaire).

All samples from the colorectal cancer patients were collected after the resection, median (range) 5 weeks (3–10) afterwards in the radically resected cancer group and 5 months (1 month–8 years) afterwards in the metastatic cancer group, but before the administration of chemotherapy. All patients (n=96) had undergone right-sided hemicolectomy, left-sided hemicolectomy, or Hartmann, sigma, abdominoperineal or anterior resection. None had undergone total or subtotal colectomy. No antibiotics, enemas or laxatives had been used for at least 2 weeks prior to sampling. The study protocol was approved by the Ethics Committee at Helsinki University Central Hospital, con-

ducted in accordance with the Declaration of Helsinki, and carefully explained to the participants, who then gave their written informed consent.

Site of colorectal cancer

Colorectal cancer had been located at the cecum (0), appendix (1), ascending colon (2), hepatic flexure (3), transverse colon (4), lienal flexure (5), descending colon (6), sigmoid colon (7), rectosigmoid junction (9) and rectum (10). Locations 0–4 were considered right-sided colorectal cancer, 5–10 left-sided, 0–7 colonic cancer and 9–10 rectal cancer.

Breath methane

Duplicate expiratory breath samples were collected twice from each subject with about 1 week in between before the patients began chemotherapy for cancer. They were collected into plastic bags, stored in 50-ml plastic syringes and analyzed for CH_4 with gas chromatography (Quintron MicroLyzer, Model DP, QuinTron Instrument Co., Milwaukee, WI, USA) within 4 days. The gas was determined to have been preserved for 4 days with only 10% reduction in concentration. The subject was diagnosed as being a CH_4 producer if there was 3 ppm or more CH_4 in two of the total four syringes.

Fecal methane and pH

The subjects provided fecal samples for CH_4 analysis near the time of the breath samples and were instructed to refrigerate them until transport to the investigators. Once received, the samples were analyzed for pH with a glass electrode. The time from defecation to analysis ranged between 2 and 72 h. Fecal samples were either analyzed immediately for CH_4 or stored at −70°C before analysis.

A modification of the method described by Ross [20] and Ross and Shaffer [21] was used to measure CH_4 production by fecal microbiota in vitro. In order to prepare fecal suspensions (0.2% w/v), the samples were mixed with peptone yeast extract broth (PY) or peptone yeast extract with 1% glucose (PYG) in an anaerobic cabinet filled with mixed gas (90% N_2, 5% H_2, and 5% CO_2). PYG was used from the beginning of the study, whereas PY use was begun somewhat later when shown to be superior in comparison to PYG. The suspensions were incubated at 35°C for 48 h (in an anaerobic atmosphere) in strictly gas-impermeable bottles, sealed with butyl rubber caps and kept upside down during incubation and storage to prevent any loss of gas. All determinations were done in triplicate. The gas samples were taken from the vial headspace with a gas-tight syringe (2.5 ml) and analyzed for CH_4 by gas chromatography with a thermal conductivity detector (Hewlett-Packard GC model 5890 with stainless steel columns, Porapak N and Molecular Sieve, carrier gas helium, injection temperature 150°C, oven temperature 45°C, detector temperature 200°C).

Gastrointestinal symptoms

Abdominal discomfort was assessed before sampling by posing questions on flatulence, borborygmi, bloating and dyspepsia during the past 6 months to colorectal cancer patients only. Bowel movements, diarrhea and constipation were not assessed because confounding factors, related to the primary tumor and operation, were present. Symptoms were rated according to the Common Toxicity Criteria of the National Cancer Institute of Canada, scale version 2. Abdominal discomfort was graded according to the highest grade of any of the four symptoms. Colorectal cancer patients with gastrointestinal diseases, such as colitis, gluten intolerance, previous debilitating gastrointestinal operations, and symptomatic carcinomatosis, were excluded from the study. A structured questionnaire was used to check the gastrointestinal symptoms of the healthy controls when they gave their consent, and only symptom-free subjects were enrolled in the study.

Statistical analysis

Three study groups were formed using a case–control study design. The radically resected group subjects were identified first as cases, and the metastatic cancer group and healthy controls were individually matched by gender and age (±5 years). In spite of matching and paired observations, the study groups were analyzed as independent groups. Breath CH_4, fecal CH_4 production and fecal pH were the primary variables. A Chi-squared test was used to compare the groups with respect to the proportion of CH_4 producers and other dichotomous variables. The distribution of CH_4 production was skewed to the right and the values were logarithmically transformed before analysis. Before that, the values below the detection limit were transformed to the observed minimum value/2. Analysis of variance (ANOVA) was used to compare the groups with respect to continuous variables, and the results were given as means or geometric means with a 95% confidence interval (95% CI). In cases of significant global p-values, multiple comparisons were performed and the p-values were Bonferroni-corrected. Pearson correlation coefficient was used to test the linear association. Sensitivity and specificity were calculated to evaluate fecal PY and PYG methods in comparison with breath test, which was considered as a gold standard. The 95% CIs were calculated using the exact binomial formula. Kappa coefficients were also calculated to evaluate agreement between the different methods. All tests were two-sided, and P-values <0.05 were

Table 1 Characteristics of study subjects

	Colorectal cancer Total n=96	Radically resected colorectal cancer n=48	Metastatic colorectal cancer n=48	Healthy controls n=48
Age, median (range)	59 (47–79)	59 (49–76)	59 (47–79)	57 (43–70)
Gender, *n* (%)				
Male	52 (54%)	26 (54%)	26 (54%)	26 (54%)
Female	44 (46%)	22 (46%)	22 (46%)	22 (46%)
Cancer site, *n* (%)				
Colon	42 (44%)	19 (40%)	23 (48%)	
Rectum	54 (56%)	29 (60%)	25 (52%)	
Right[a]	15 (16%)	6 (12%)	9 (19%)	
Left[b]	81 (84%)	42 (88%)	39 (81%)	
Surgical procedure, *n* (%)				
Right hemicolectomy	14 (15%)	5 (10%)	9 (19%)	
Left hemicolectomy	13 (13%)	5 (10%)	8 (17%)	
Sigma resection	14 (15%)	9 (19%)	5 (10%)	
Hartmann procedure	2 (2%)	1 (2%)	1 (2%)	
Anterior resection	41 (43%)	21 (44%)	20 (42%)	
Abdominoperineal resection	12 (12%)	7 (15%)	5 (10%)	
Stomas	21 (22%)	13 (27%)	8 (17%)	

[a]Proximal to splenic flexure

[b]Distal to splenic flexure

considered significant. Statistical analyses were performed with the StatView computer program (version 5.0.1; SAS Institute Inc., Cary, NC, USA) and SPSS statistical software (version 15.0; SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

Subject characteristics were well balanced between the groups (Table 1). Two healthy subjects and two metastatic cancer patients failed to provide fecal samples.

Method comparison

Results attained by PY and PYG methods were in good agreement with each other (89% agreement, Kappa 0.77). However, PYG categorized as nonproducers 26% of those who were categorized as CH_4 producers by PY. When compared to the results from the breath test, PY was far more sensitive than PYG [sensitivity (95% CI) 0.96 (0.80–1.00) versus 0.48 (0.33–0.63)]. Results by PYG were in moderate agreement with the breath test results (73% agreement, Kappa 0.45), whereas results by PY were in good agreement with the breath test results (90% agreement, Kappa 0.77).

Table 2 Methane producers by different assessment methods and fecal pH in study groups

	Breath test		In vitro[c]		Fecal pH	
	All n[a]	Methane producers[b] n (%)	All n	Methane producers[b] n (%)	n	Mean (95% CI)[d]
Colorectal cancer total	96	44 (46%)	58	21 (36%)	92	6.77 (6.63–6.91)
Radically resected colorectal cancer	48	22 (46%)	30	11 (37%)	48	6.77 (6.56–6.99)
Metastatic colorectal cancer	48	22 (46%)	28	10 (36%)	46	6.76 (6.59–6.94)
Healthy controls	48	19 (40%)	30	12 (40%)	46	6.80 (6.66–6.94)
P		0.776		0.938		0.954

[a] Number of samples analyzed

[b] Chi-squared test (radically resected cancer versus metastatic cancer versus healthy)

[c] [PY peptone yeast extract broth]

[d] ANOVA (radically resected cancer versus metastatic cancer versus healthy)

Methane production

There were no significant differences in the proportion of CH_4 producers between the study groups (Table 2). The geometric means (95% CI) of breath CH_4 in the healthy, the radically resected and the metastatic cancer groups, respectively, were 0.90 ppm (0.48–1.69), 1.12 ppm (0.56–2.25) and 1.01 ppm (0.53–1.91) (P=0.898). The individual breath CH_4 values are presented in Fig. 1. There were significantly more participants with breath CH_4 20 ppm or more in the radically resected group (22.9%) than in the healthy control group (4.2%) [OR (95% CI) 6.8 (1.4–32.8), P=0.016].

Fewer CH_4 producers were found among patients with resected right-sided cancer than among those with resected left-sided cancer (Table 3). The proportion of CH_4 producers was also lower among colon cancer patients with right-sided hemicolectomy than among those with other surgical procedures (Table 3). CH_4 producers did not differ from nonproducers regarding the presence of stoma or the time from the operation to the analysis (data not shown).

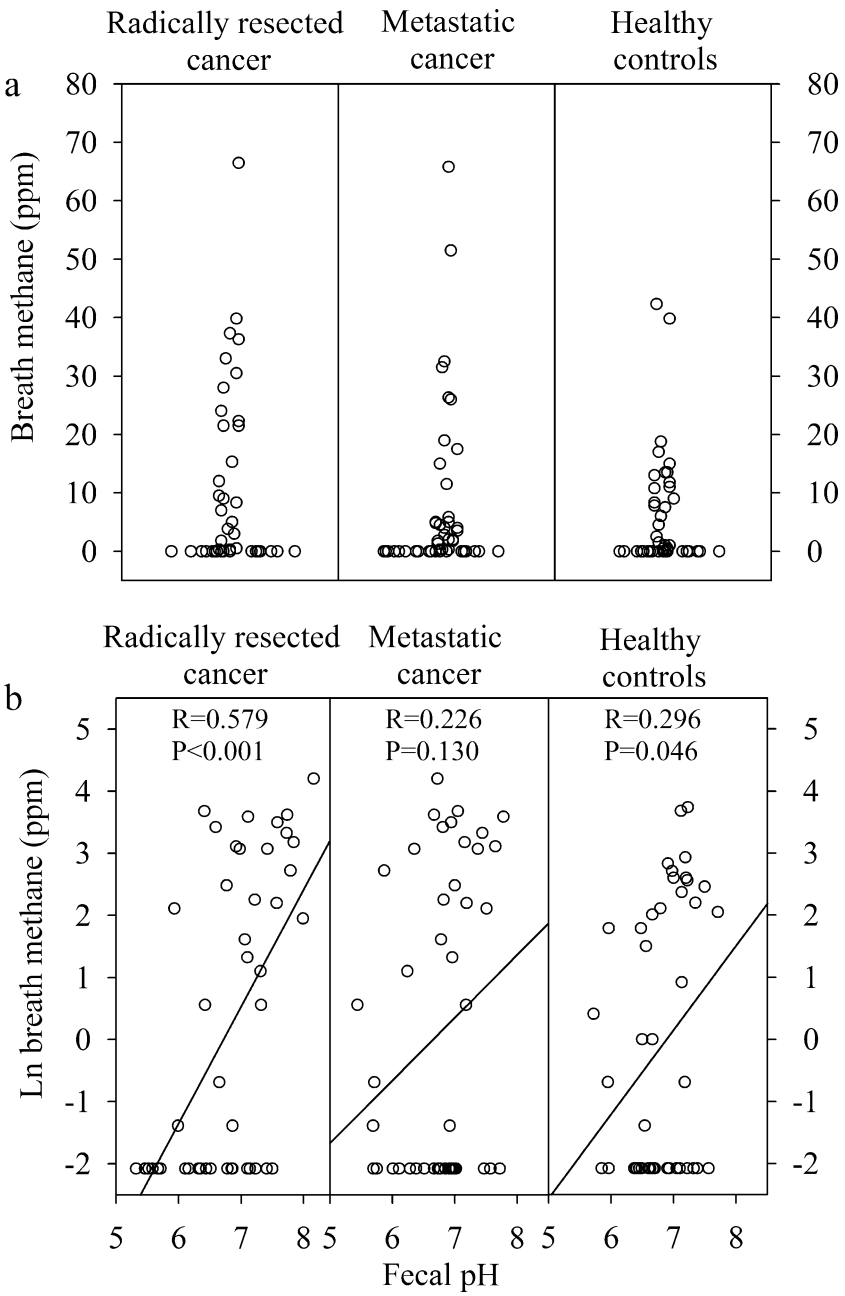

Fig. 1 **a** Individual values of breath methane in the study groups. n=48 in each group. **b** Correlation between breath methane and fecal pH within each study group. Radically resected cancer n=48, metastatic cancer n=46, healthy controls n=46

Table 3 Methane producers and fecal pH according to the site of cancer and surgical procedure

	Breath test		Fecal pH	
	All n[a]	Methane producers[b] n (%)	Number of samples	Mean (95% CI)[c]
Cancer site				
Colon	42	19 (45%)	40	6.66 (6.45–6.87)
Rectum	54	25 (46%)	54	6.85 (6.67–7.03)
P		0.918		0.177
Right[d]	15	3 (20%)	14	6.27 (5.92–6.62)
Left[e]	81	41 (51%)	80	6.86 (6.72–7.00)
P		0.029		0.002
Surgical resection				
Right hemicolectomy	14	2 (14%)	13	6.19 (5.92–6.62)
Left hemicolectomy, Hartmann or sigma	29	18 (62%)	28	6.93 (6.61–7.14)
Abdominoperineal or anterior	53	24 (45%)	53	6.83 (6.68–7.02)
P		0.013		0.002

[a]Number of samples analyzed
[b]Chi-squared test
[c]ANOVA
[d]Proximal to splenic flexure
[e]Distal to splenic flexure

Fecal pH

Fecal pH did not differ between healthy subjects and colorectal cancer patients with radically resected cancer or metastatic cancer (Table 2). Fecal pH was significantly lower in patients with resected right-sided cancer than in those with resected left-sided cancer (Table 3) and in healthy subjects [6.80 (6.66–6.94), P=0.010]. Also, colon cancer patients with right-sided hemicolectomy had lower fecal pH than those with other resection types (Table 3).

Fecal pH was significantly higher in CH_4 producers than in nonproducers according to both CH_4 methods [7.05 (6.92–7.19) versus 6.57 (6.44–6.70), P<0.001, when categorization was based on the breath tests]. Breath CH_4 values and fecal pH were positively correlated (R=0.396, P<0.001, n=140, Fig. 1). When excluding patients with right-sided hemicolectomy and thus lower fecal pH (n=14), fecal pH was also significantly higher in CH_4 producers than in nonproducers according to both CH_4 methods [7.08 (6.94–7.21) versus 6.64 (6.50–7.77), P<0.001, when categorization was based on the breath tests], and breath CH_4 values and fecal pH were positively correlated (R=0.382, P<0.001, n=127).

Abdominal discomfort

Abdominal discomfort data (flatulence, borborygmi, bloating and dyspepsia) was available from all resected colorectal cancer patients. Forty-three percent of the colorectal cancer patients had abdominal discomfort, 50% in the radically resected cancer group and 35% in the metastatic cancer group (P=0.149). Abdominal discomfort did not significantly differ between different tumor sites and surgical procedures (data not shown). Breath CH_4 excretors had significantly less abdominal discomfort than nonexcretors (30% versus 54%, P=0.016). Also, patients with abdominal discomfort had a lower breath level of CH_4 than patients without abdominal discomfort [geometric mean (95% CI): 0.57 ppm (0.28 to 1.13) versus 1.70 ppm (0.92 to 3.14), P=0.019].

Discussion

Forty-six percent of patients with resected colorectal cancer were breath CH_4 excretors in the present study. This is similar to the previously reported breath CH_4 excretor rates of 47% and 51% in resected colorectal cancer patients [13, 22]. Of healthy subjects, 40% were breath CH_4 excretors. This is in line with the results obtained from several large studies, where 34–48% of healthy adults were found to excrete CH_4 in breath [7, 12, 23, 24]. In concordance with the results of the previous studies [13, 22], there were no significant differences in the present study in the number of CH_4 producers between the resected colorectal cancer patients and the healthy subjects. Individuals' microbiota composition is usually considered to remain constant except for fluctuations due to antibiotic treatment [1], and thus, in most patients in the present study, the methane producer status probably reflects the status before resection. The tumor itself, however, may increase CH_4 production by obstruction [5], and its absence after resection may have turned some CH_4 excretors into nonexcretors.

There were no differences in fecal pH between the resected colorectal cancer patients and the healthy subjects. This finding is similar to two previous reports of no differences in intraluminal and fecal pH between patients

with colorectal cancer and healthy subjects [25, 26]. However, high fecal pH has been detected in patients with colorectal cancer (mainly distal disease) in an earlier study and in patients with resected sigmoid colon cancer [18, 19].

A positive association between fecal pH and CH_4 excretion was found in the present study, in line with previous studies in vitro and in healthy adults [14, 27]. Interestingly, fecal pH has been reported as not differing between healthy CH_4 excretors and nonexcretors consuming a conventional diet, but when consuming lactulose, fecal pH is lower in nonexcretors [28]. Therefore, methanogenesis does not seem to result merely from higher colonic pH. Low fecal butyrate concentrations have been linked with high numbers of methanogens, and the possible explanation could be that methanogens outcompete acetogens leading to the lack of acetate and hence also butyrate [3, 27].

Resected right-sided cancer and right-sided hemicolectomy were associated with reduced CH_4 production and fecal pH. It is unlikely that bile acids were responsible for these reductions, since a previous study detected no differences in total fecal bile acid excretion after right- or left-sided hemicolectomy in comparison with control subjects with intact colon [29]. Fecal pH after right hemicolectomy has been reported as not differing from fecal pH in an intact colon [29]. Therefore, low methanogenesis and fecal pH are possibly markers of colonic microbiota that is characteristic of right-sided colorectal cancer, but because this observation is based on a limited number of patients, no firm conclusion can be drawn.

In this study, CH_4 producers had less abdominal discomfort than nonproducers. This is in agreement with previous studies where breath CH_4 excretors had a lower incidence of symptoms characteristic of lactose intolerance, such as gaseousness and abdominal pain [24, 30]. In CH_4 producers, fecal hydrogen, the major gas produced by intestinal fermentation, is much more rapidly consumed than in nonproducers [7, 30]. This results in decreased gas volumes since one volume of CH_4 is produced from four volumes of H_2 [31]. Thus, these results support the hypothesis that methanogenesis is an important resource for hydrogen gas disposal in vivo and helps alleviate abdominal discomfort due to excess gas production [6].

In conclusion, the results of the present study do not indicate that patients with resected colorectal cancer as a whole would differ in their colonic methanogenesis or pH from healthy subjects with intact colon. However, low methanogenesis as well as low fecal pH were characteristic of right-sided colorectal cancer. Irrespective of resected tumor site, increased abdominal discomfort appears to be associated with low methanogenesis in resected colorectal cancer patients.

Acknowledgements We thank T. Poussa for helping with the statistics. This study was funded by Valio Ltd., Helsinki, Finland. There is no potential conflict of interest.

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