Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls

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ABSTRACT

Background: Patients with Parkinson's disease (PD) frequently have gastrointestinal symptoms (e.g. constipation) and exhibit the PD-typical pathohistology in the enteric nervous system (ENS). Both, clinical symptoms and pathohistological changes in the ENS occur at early stages and can precede the motor manifestations of PD. Two recent studies reported an association between changes in gut microbiota composition and PD. We hypothesized that alterations in gut microbiota might be accompanied by altered concentrations of short chain fatty acids (SCFA), one main metabolic product of gut bacteria.
Methods: We quantitatively analyzed SCFA concentrations (using gas chromatography) and microbiota composition (using quantitative PCR) in fecal samples of 34 PD patients and 34 age-matched controls.
Results: Fecal SCFA concentrations were significantly reduced in PD patients compared to controls. The bacterial phylum *Bacteroidetes* and the bacterial family *Prevotellaceae* were reduced, *Enterobacteriaceae* were more abundant in fecal samples from PD patients compared to matched controls.
Conclusions: Our study confirms the recently reported association between PD and the abundance of certain gut microbiota and shows a reduction in fecal SCFA concentrations (one main metabolic product of certain gut bacteria). The reduction in SCFA might, theoretically, induce alterations in the ENS and contribute to gastrointestinal dysmotility in PD. Prospective longitudinal studies in subjects at risk for PD are required to further elucidate the causal role of gut microbiota and microbial products in the development of PD and PD-associated dysmotility.

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder clinically characterized by motor and non-motor symptoms. The pathohistological hallmark of PD is the presence of aggregated proteins (Lewy bodies) in the nervous system. Lewy body pathology in PD is found in structures of the central nervous system (CNS) as well as in the peripheral autonomic [1] and enteric nervous system (ENS) [2–5]. PD patients frequently exhibit non-motor symptoms, including signs and symptoms of gastrointestinal dysmotility (e.g.

delayed gastric emptying [6–8], constipation [9,10]). Lewy body pathology in the ENS might represent the pathohistological correlate for gastrointestinal symptoms in PD. Current hypotheses suggest that the ENS might be one of the first sites where Lewy body pathology appears in PD [2,11]. Interestingly, an animal model of PD shows that chronic intragastric administration of rotenone, a complex-1-inhibitor, results in a spatio-temporal distribution of alpha-synuclein immunoreactivity that is compatible with the above mentioned hypotheses [12,13]. A similar trigger might be responsible for the formation of Lewy bodies in humans.

Due to the immediate vicinity of the ENS with feces, gut microbiota and microbiota's metabolic products are among potential candidates that could initiate a process that eventually results in the formation of Lewy bodies in the ENS. Two recent studies

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showed an association between the abundance of certain gut microbiota and PD [14,15]: amongst others, bacteria that have the capacity to produce short chain fatty acids (SCFA) were reduced in PD. SCFA modulate the activity of the ENS and thereby increase gastrointestinal motility [16]. Hence, altered concentrations of SCFA might contribute to gastrointestinal dysmotility in PD.

We hypothesized that a shift in gut microbiota might be associated with a shift in gut microbiota's metabolic products, e.g. SCFA. We therefore analyzed microbiota and SCFA in fecal samples of patients with PD and age-matched healthy controls.

2. Subjects and methods

This study was approved by the Ethics committee of the medical association of Saarland. All enrolled subjects provided written informed consent.

2.1. Subjects

All subjects (patients as well as controls) were on an omnivorous diet and none of the subjects reported special dietary habits or dietary restrictions (e.g. due to food intolerance). None of the subjects reported intake of antibiotic drugs or intake of probiotic or prebiotic products during the last 3 months. None of the enrolled subjects had a history of acute or chronic gastrointestinal disorder. PD patients (24 male, 10 female) were diagnosed according to UK PD Society Brain Bank Clinical Diagnostic Criteria by a movement disorder specialist. All 34 PD patients were on dopaminergic drugs. Mean disease duration for PD (defined by the time the first motor symptoms were experienced by the patient) was 82 months (range: 12—228 months). Median Hoehn and Yahr stage was 2.5 (range: 1 to 4). All PD patients were interviewed for autonomic symptoms (including constipation and symptoms of gastrointestinal discomfort), pre-existing gastrointestinal disorders and previous surgical interventions on the gastrointestinal tract (individual data of all PD patients are provided in Supplemental Table 1). Seven of the 34 PD patients reported constipation. Mean age in the PD patient group was 67.7 ± 8.9 years. The fecal samples of PD patients were age-matched to samples of healthy controls (HC, n = 34, 18 male, 16 female) from the repository of the Institute of Microoecology in Herborn (mean age of HC 64.6 ± 6.6 years). None of the control subjects reported preexisting medical conditions. None of the control subjects was on chronic or intermittent use of drugs affecting gastrointestinal motility or on any other permanent medication. Two of the 34 control subjects reported constipation. No other gastrointestinal symptoms were reported by the control subjects. In order to identify age-related changes we also included a small group of younger healthy controls (n = 10, 5 male, 5 female, 33.3 ± 11.6 years). Additional information is provided online (Supplemental Table 1 and Supplemental Material).

2.2. Collection of fecal samples

All subjects were provided with sterile containers and instructed how to collect the fecal samples at home and how to send the samples to the Institute of Microoecology, Herborn. The stool samples were immediately frozen at - 35 °C and analyzed within a few days.

2.3. Sample preparation and DNA extraction

Microbial DNA was extracted using the QIAsymphony® DSP Virus/Pathogen Mini-Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Automated isolation and pipetting of 96-well plates (Micro Amp Optical 96-Well Reaction Plate,

Applied Biosystems, Darmstadt, Germany) were performed by the QIAsymphony® SP/AS instrument (QIAGEN, Hilden, Germany) using the QIAsymphony DSP Virus/Pathogen Mini-Kit.

2.4. Quantification of target bacteria by real-time quantitative PCR (qPCR)

Primers were selected to recognize either the whole bacterial phylum (Firmicutes, Bacteroidetes) or main representatives of a phylum (*Akkermansia muciniphila*, the genus *Bifidobacterium, Enterobacteriaceae, Methanobrevibacter smithii*). In addition, the genera *Lactobacillus* and *Lactococcus* were enumbered. PCR amplification and detection was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Darmstadt, Germany) in optical-grade 96-well plates. A standard curve was produced using the appropriate reference organism to quantify the qPCR values into number of bacteria/g. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was carried out following amplification to distinguish the targeted PCR product from the non-targeted PCR product. The melting curves were obtained by slow heating at temperatures from 55 to 95 °C at a rate of 0.2 °C/s, with continuous fluorescence collection. The data was analyzed using the ABI Prism software. The real-time PCRs were performed in triplicate.

2.5. Determination of SCFA concentrations

Fecal samples were lyophilized and analyzed using gas chromatography. The lyophilisate was dissolved in 100 μl 5 M HCOOH and 400 μl Aceton and centrifuged (5 min at 4000 × g). Concentrations of SCFA were determined in the supernatant using a GC-2010 Plus gas chromatograph (Shimadzu Deutschland GmbH, Duisburg, Germany) equipped with a flame ionization detection with a thin-film capillary column Stabilwax®-DA 30 m × 0,25 mm × 0,5 μm (Restek, Bad Homburg, Germany). The samples were spread out by split injection using the auto-sampler AOC-20s/I (Shimadzu Deutschland GmbH). GCsolution Chromatography Data System (Shimadzu Deutschland GmbH) was used for data processing. An external standard (Supelco™ WSFA-1 Mix, Supelco Sigma-Aldrich Co., Bellefonte PA) was used for quantification of SCFA.

2.6. Statistical analysis

Statistics was carried out using GraphPad Prism 6.0 and PASW statistics software version 22.0 (SPSS Inc., Chicago, IL, USA). Data are displayed as mean ± standard error of the mean. D'Agostino-Pearson omnibus normality test and Shapiro-Wilk normality test were applied to analyze Gaussian distribution. The Kruskal-Wallis test was applied to analyze global differences between the three groups. Mann-Whitney *U* test was used for post-hoc comparison. In order to control family-wise error rate caused by multiple comparison Bonferroni correction was carried out. Correlation was investigated using nonparametric Spearman correlation with a two-tailed confidence interval (95%). Binary logistic regression was performed to investigate the relationship between constipation and microbiota, SCFA concentrations respectively (all bacteria and SCFA were integrated in the binary logistic regression model).

3. Results

The percentage of bacterial phyla and bacterial groups in fecal samples of PD patients and age-matched controls were determined by quantitative PCR. Results are shown in Fig. 1: The bacterial phylum *Bacteroidetes* was significantly reduced in fecal samples of
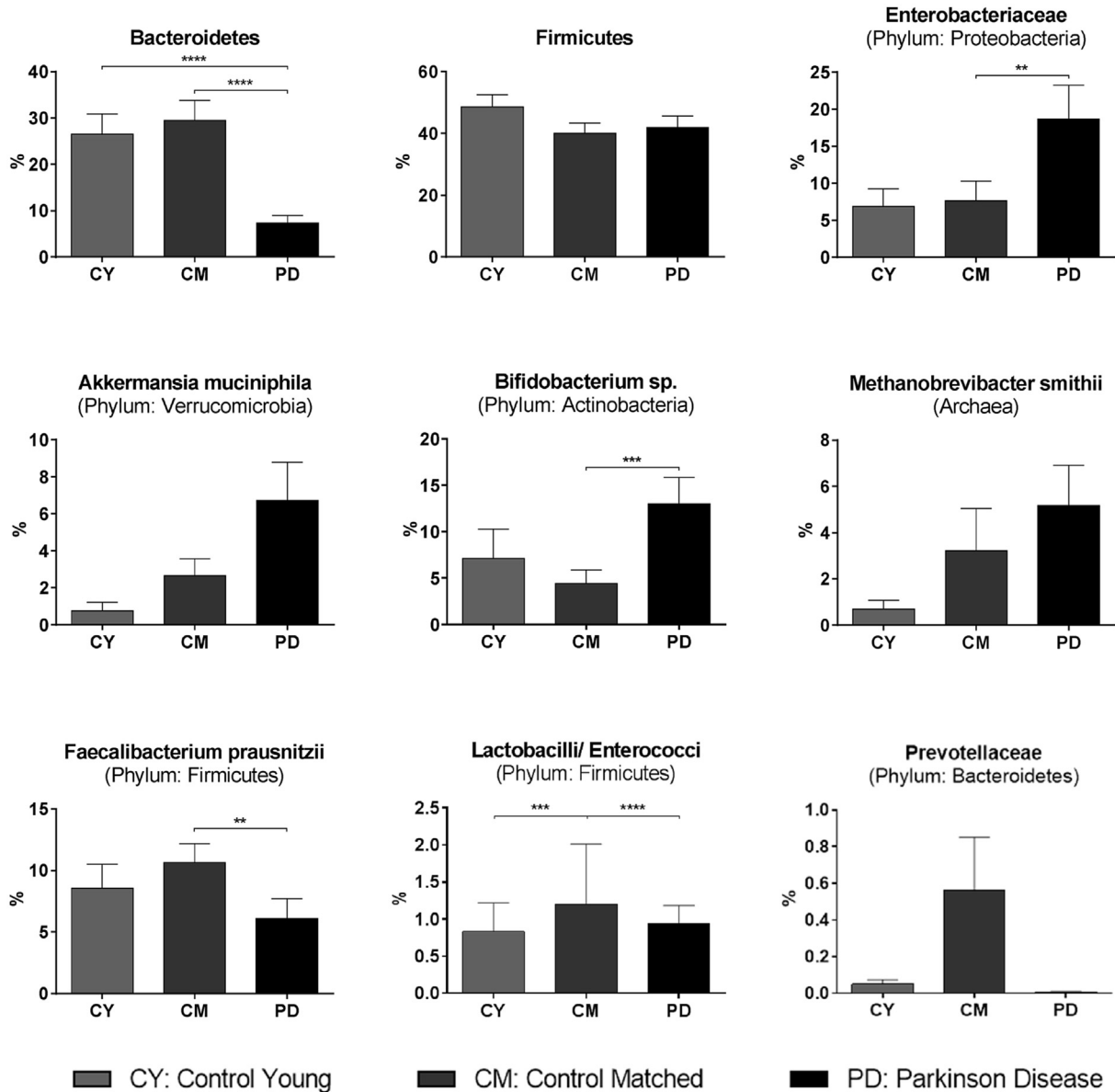
Fig. 1. *Gut microbiota composition*. Figure 1 shows the relative numbers for two bacterial phyla (*Bacteroidetes* and *Firmicutes*) as well as relative numbers for main representatives of bacterial phyla in fecal samples of PD patients (*PD*, mean age 67.7 ± 8.9 years), aged-matched healthy controls (*CM*, mean age 64.6 ± 6.6 years) as well as group of young controls (*CY*, mean age 33.3 ± 11.6 years). The latter group was included to identify age-related alterations. **p < 0.01; ***p < 0.001; ****p < 0.0001.

PD patients. *Prevotellaceae* (a bacterial family assigned to the phylum *Bacteroidetes*) were also descriptively reduced in PD patients compared to controls.

The phylum *Firmicutes* was the most abundant phylum and showed a similar relative abundance in all groups. However, *Faecalibacterium prausnitzii* (a bacterial species that belongs to the phylum *Firmicutes*) as well as *Lactobacillaceae* and *Enterococcaceae* (two bacterial families that belong to the phylum Firmicutes) were significantly reduced in the samples of PD patients compared to age-matched controls.

The bacterial species *Akkermansia muciniphila* (non-significant), and the genus *Bifidobacterium* (p < 0.001) as well as the bacterial family *Enterobacteriaceae* (p < 0.01) were more abundant in PD patients compared to controls. The abundance of *Enterobacteriaceae* did not differ between clinical PD phenotypes (classified as tremor-dominant, hypokinetic-rigid, or mixed type; Supplemental Fig. 3). *Methanobrevibacter smithii* (the predominant representative

of *Archaea* in humans) showed a similar relative abundance in the samples of PD patients compared to age-matched controls, but a reduced abundance in younger controls.

Quantitative analysis of SCFA concentrations in fecal samples revealed a significant decrease in absolute concentrations for acetate, propionate and butyrate (Fig. 2a) and also a significant relative reduction for butyrate (Fig. 2b) in PD patients compared to age-matched controls. The reduction in SCFA concentrations in fecal samples of PD patients exceeded the age-related decline for acetate, propionate and butyrate concentrations. Valerate, iso-valerate as well as iso-butyrate concentrations did not differ between the fecal samples of PD patients and age-matched controls.

In order to identify possible relationships between microbiota, SCFA concentrations respectively, and clinical parameters, we performed logistic regression and correlation analyses. Logistic regression analysis did not reveal a relationship between the presence of constipation and the abundance of any of the
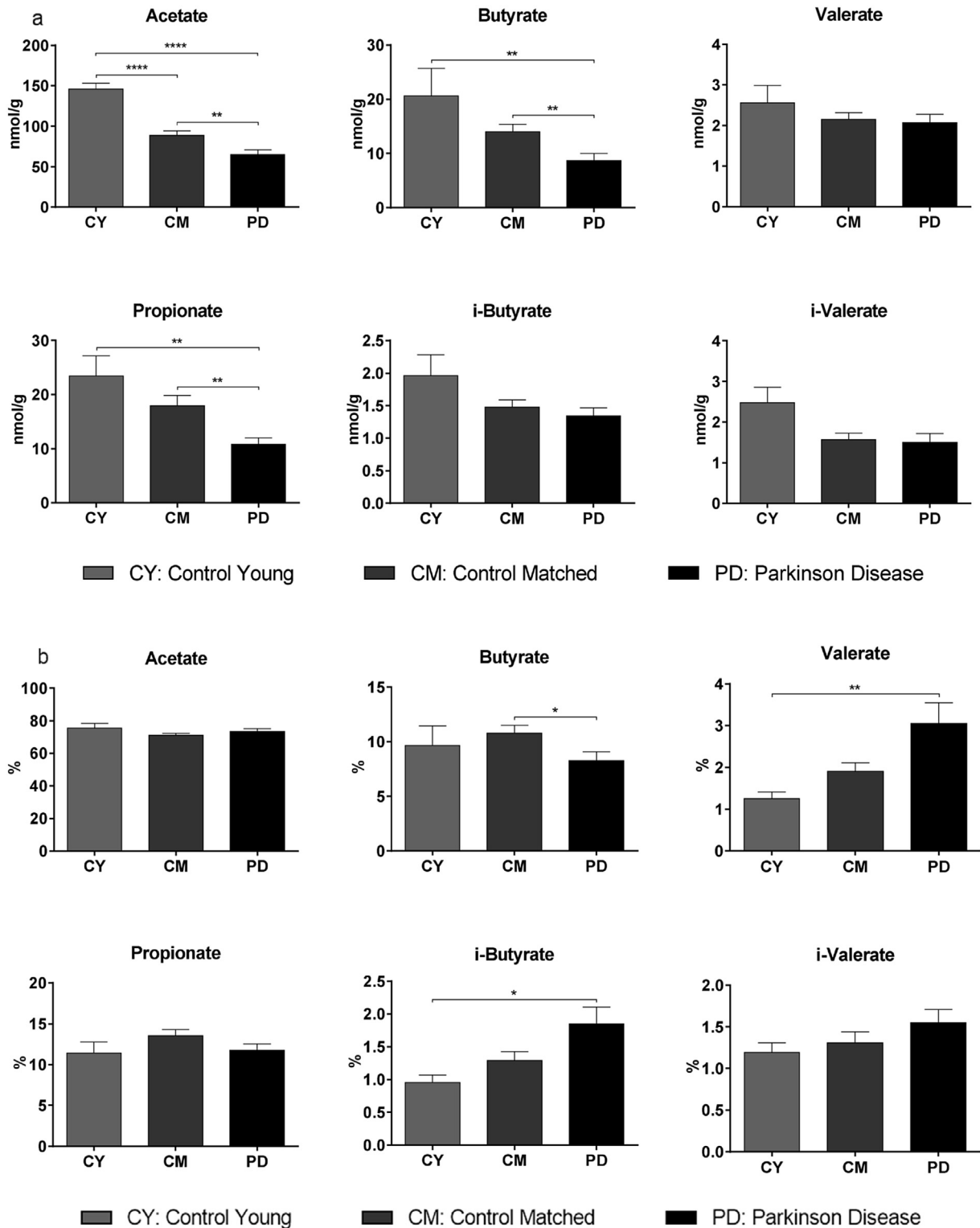
Fig. 2. a. *Absolute concentrations of short chain fatty acids*. Figure 2a shows the absolute concentrations (mean + standard error of the mean in mmol/g) for the short chain fatty acids *butyrate, iso-butyrate, valerate, iso-valerate, propionate and acetate* in fecal samples of PD patients (*PD*, mean age 67.7 ± 8.9 years), aged-matched healthy controls (*CM*, mean age 64.6 ± 6.6 years) as well as group of young controls (*CY*, mean age 33.3 ± 11.6 years). The latter group was included to identify age-related alterations. $^*p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$. b. *Relative concentrations of short chain fatty acids*. Figure 2b shows the percentage (mean + standard error of the mean) of the short chain fatty acids *butyrate, iso-butyrate, valerate, iso-valerate, propionate and acetate* in fecal samples of PD patients (*PD*, mean age 67.7 ± 8.9 years), aged-matched healthy controls (*CM*, mean age 64.6 ± 6.6 years) as well as group of young controls (*CY*, mean age 33.3 ± 11.6 years). The latter group was included to identify age-related alterations. $^*p < 0.05$; $^{**}p < 0.01$.

investigated microbiota, SCFA concentrations respectively, in PD. Concerning medication, use of entacapone negatively correlated

with the abundance of *Firmicutes* (correlation coefficient −0.448, p 0.008) and the abundance of the bacterial species *Faecalibacterium*

prausnitzii (correlation coefficient −0.510, p 0.002). Additionally, use of entacapone showed a negative correlation for absolute (correlation coefficient −0.389, p 0.023) as well as for relative (correlation coefficient −0.452, p 0.007) butyrate concentrations. Additional information concerning possible relationships between microbiota, SCFA concentrations respectively, and clinical parameters is provided online as Supplemental File.

4. Discussion

Gastrointestinal symptoms as well as Lewy body pathology in the ENS occur at early stages of PD. Current hypotheses discuss the gut as the origin of the pathological process that underlies PD [11]. Gut microbiota and their metabolic products are among potential candidates that could ignite a process that eventually leads to Lewy body formation in the ENS. Two recent studies showed that the abundance of certain gut microbiota differs between PD patients and controls [14,15].

Here we report alterations in gut microbiota composition that reproduce some of these previously reported findings. We used a different methodological approach (quantitative PCR) that rather focused on re-assessing previously reported data in an independent sample of PD patients than analyzing (sequencing) the whole fecal microbiome. We did not use nutrition diaries, but all subjects were interviewed for dietary habits and were explicitly asked for special dietary habits or dietary restrictions. Since all subjects reported a Central-European omnivorous diet without any dietary restrictions, dietary habits are unlikely to be a major confounder in the investigated subjects.

Our data are in accordance with previously reported findings: Similar to the results reported by Scheperjans et al. [14] we observed a (non-significant) relative reduction in *Prevotellaceae*. *Enterobacteriaceae* were more abundant in fecal samples of PD patients. Interestingly, Scheperjans and colleagues reported that *Enterobacteriaceae* were more abundant in PD with a PIGD (postural instability and gait difficulty) phenotype compared to tremor-dominant PD patients [14]. In our study, we observed an overall increased abundance of *Enterobacteriaceae* in PD patients compared to healthy controls (p < 0.01). The abundance of *Enterobacteriaceae* did not differ between PD phenotypes (classified as tremor-dominant, hypokinetic-rigid, or mixed type, p 0.5067, Supplemental Fig. 3). When PD patients with the hypokinetic-rigid and mixed type were grouped and compared to tremor-dominant PD patients, there was still no statistically significant difference (p 0.5813) concerning the abundance of *Enterobacteriaceae*. This discrepancy concerning PD phenotype and abundance of *Enterobacteriaceae* between the work of Scheperjans and colleagues and our study might be in part due to the small number of tremor-dominant PD patients in our sample (n = 6).

Forsyth et al. reported an increased intestinal permeability in PD that correlated with staining for *E. coli* (a bacterial species assigned to the family *Enterobacteriaceae*) and concluded that a compromised intestinal barrier might help to expose the ENS to noxa in PD [17]. Hence, the observed increased abundance of *Enterobacteriaceae* (we did not specifically analyze the abundance of the bacterial species *E. coli*, but the corresponding bacterial family of *Enterobacteriaceae*) might be pathophysiologically relevant for PD. In contrast to Scheperjans and colleagues [14] we observed a reduction in *Lactobacillaceae*. Anti-inflammatory effects of some *Lactobacillaceae* would be compatible with the above mentioned hypothesis of a reduction in potentially beneficial bacteria in PD. Other studies are in line with the hypothesis of an altered intestinal epithelial barrier and pro-inflammatory process in PD: a reduced expression of occludin (a tight junction protein) and a morphologically altered intestinal epithelial barrier [18] as well as an increased mRNA expression of pro-inflammatory cytokines [19] have been shown in colonic biopsies from PD patients.

In contrast to the results from Keshavarzian et al. (who reported a significantly increased abundance of *Bacteroidetes* and a significantly reduced abundance of Firmicutes [15]) we observed a reduction in the phylum *Bacteroidetes* and no significant differences between PD patients and matched controls for the phylum *Firmicutes*. Differences in mean age, disease duration as well as medication status (approximately one third of the PD patients in the study by Keshavarzian et al. was drug-naïve, while all of the patients enrolled in this study were on dopaminergic treatment) might account for this difference. Compatible with the results by Keshavarzian et al. [15], we observed an increased abundance in *Enterobacteriaceae* (phylum: *Proteobacteria*). The reduction in the abundance of *Faecalibacterium prausnitzii* and *Prevotellaceae* we observed in this study is also compatible with results Keshavarzian and colleagues reported for the investigated mucosa samples.

We found a significant reduction of acetate, propionate and butyrate in fecal samples of PD patients compared to healthy controls. For butyrate, not only the absolute but also the relative concentrations were significantly reduced in PD patients. Butyrate acts locally on the colonic mucosa but can also exert remote effects via the ENS, e.g. butyrate alters the activity of enteric neurons by reversible hyperpolarization [20].

Interestingly, sodium-butyrate, a histone deacetylase inhibitor (HDACi), protects dopaminergic neurons [20,21] and prevents motor impairment in a toxin-induced drosophila model of PD [22]. It would be interesting to investigate HDACis in clinical trials to clarify whether such an epigenetic approach affects gastrointestinal dysfunction in PD or even the neurodegenerative process itself. Butyrate has also been shown to interact with the ENS and to increase colonic contractility [16]. In critically ill subjects, lower concentrations of butyrate are associated with dysmotility [23]. Taken together, these data are compatible with the assumption that reduced concentrations of butyrate in the feces of PD patients might exert relevant effects on the ENS and might contribute to gastrointestinal dysmotility, a frequent non-motor symptom in PD. The reduction in SCFA and the reduction *in Faecalibacterium prausnitzii* seen in our study is compatible with Keshavarzian's study that investigated microbiota in sigmoid mucosal biopsies and found a reduction in butyrate-producing bacteria in PD including *Faecalibacterium prausnitzii* [15]. Anti-inflammatory effects and beneficial effects on the intestinal epithelial barrier have been reported for *Faecalibacterium prausnitzii* [24,25]. Hence, a reduction in *Faecalibacterium prausnitzii* in combination with an increased abundance of *Enterobacteriaceae* (see above) might compromise the intestinal epithelial barrier and thereby make the ENS more susceptible to intraluminal pathogens.

Concerning the effect of medication on microbiota, we identified a strong negative correlation between the use of entacapone and the abundance of *Firmicutes* and *Faecalibacterium prausnitzii*. Entacapone also showed a negative correlation with fecal butyrate concentrations. Butyrate is a putative metabolic product of *Faecalibacterium prausnitzii*. Hence, though our data do not prove causality between medication, microbiota and SCFA, the identified correlations are plausible and endorse the need for future studies that focus on the interaction between drugs, microbiota and microbial products.

We did not identify a relationship between the presence of constipation in PD and distinct microbiota, SCFA concentrations respectively. Yet, this aspect needs to be re-evaluated in future studies, as the prevalence of constipation in our patient sample was rather low (only 7 of the 34 enrolled PD patients reported presence of constipation).

Concerning the group of younger controls, the results should be

considered preliminary due to the small sample size (n = 10). Nevertheless, the data are of interest due to the fact that there is sparse data on age-dependent changes in gut microbiota and fecal SCFA. On a descriptive level, our data indicate age-dependent differences for certain microbiota and SCFA. The development of gut microbiota composition and SCFA concentrations in ageing should be investigated in further studies to understand whether PD (and other neurodegenerative disorders) show a pattern of accelerated ageing concerning these parameters. Our preliminary data in young controls (and the differences between young and matched controls) also stress the impact of age in microbiota studies.

In summary, our study confirms the association between PD and the abundance of certain gut microbiota. Our results largely overlap with previously reported findings, despite the differences in the methodological approach and the different geographical background of the investigated subjects. In addition, we show a significant absolute and relative reduction in fecal SCFA (one main metabolic product of certain (putative beneficial) gut bacteria) that is consistent with the observed altered gut microbiota composition. In addition, the reduction in SCFA concentrations might, theoretically, contribute to constipation in PD. Our study does neither allow conclusions about a causal relationship between the abundance of certain microbiota, SCFA and PD nor conclusions concerning a causal relationship between microbiota, SCFA and constipation. Yet, given the lack of data in this field, our results together with the above mentioned studies endorse the necessity for further investigations in this field. In order to reveal a causal relationship between the abundance of certain microbiota, SCFA respectively, and PD, prospective longitudinal studies in subjects at risk for PD are required to further elucidate the role of gut microbiota and their products in the development of PD and PD-associated dysmotility. Differentiation between central and peripheral effects on gastrointestinal motility is another challenging task in microbiota studies. Studies in patients with (traumatic) spinal cord injury or pelvic nerve transection (that result in denervation of the colon) might contribute to the understanding of central versus peripheral mechanisms.

Conflict of interest/disclosure

All authors declare that there is no conflict of interest regarding this work. All authors have approved the final article.

Authors' contributions to the article

1) a) conception, design; b) data acquisition; c) analysis, interpretation.
2) a) drafting the article; b) revising the article critically.
3) a) final approval of the version to be submitted.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.parkreldis.2016.08.019.

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