Characterization of host associated microbiota under influencing factors
A case study on human gut and *Brachypodium* root

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Shaodong Wei
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Preface

This thesis is the result of my work as a PhD student in the Section of Microbiology, Department of Biology, Faculty of Science, University of Copenhagen, in addition with 3-month work (September to December, 2017) in INRA, UMR 1347 Agroécologie, Dijon, France.

I would like to thank my supervisor Professor Søren J. Sørensen for giving me the opportunity to study in his group. It has been a great experience for me to meet the people, culture, and nature in Denmark. Big thanks to Martin S. Mortensen and Asker D. Brejnrod for helping me start in data analysis with R. I am also grateful to Samuel Jacquiod for supporting me in personal life when I was working in France and the big help in manuscript preparation. I would also like to thank the COPSAC research group at Gentofte Hospital for their substantial contributions to my work. Besides, this work could not have been done without the help and support of the entire Section of Microbiology. Big thanks to Luma Odish, Jannie R. Christensen and Anette H. Løth, for their technical assistance, kindness, and patience. Thanks for everyone’s smile and laughs in the Section of Microbiology. I would also like to thank the China Scholarship Council for supporting my living expenses in Denmark.

Finally, I would like to thank my family and friends for always being with me.
Abstract

Bacteria, the early form of life to develop on Earth, are present in most of the global habits, such as soil, water, clouds, acidic hot springs, and Earth’s crust etc. Among all possible habits, the microbial communities residing at the interface with large host organisms, such as human gut and around plant roots, represent two extremely complex ecological systems. The relatively stable coexistence of microbiota with their host implies the ecological significance of holobionts, the entity comprised of the macroscopic host and its associated microbiota.

We conducted three studies to investigate the relevance of symbionts to their host health and metabolism. In two studies regarding human gut, we addressed how medication use, improved diet, and exercise impact the gut microbiota. In the third study, we investigated the *Brachypodium distachyon* rhizosphere microbiota in multiple aspects including sampling scale (traditional vs. subscale), location on the root (root sections), within and between plant variations, and effect of inoculations.

The work presented here builds on three manuscripts:

**MANUSCRIPT 1**, “Short- and long-term impacts of azithromycin treatment on the gut microbiota in children: A double-blind, randomized, placebo-controlled trial”, is a study addressing the global concerns regarding the undesired effects of antibiotics use. We focused on the influence of antibiotics treatment on gut microbiota in early life. With the strength of randomized clinical trial (RCT) study design, we showed that azithromycin led to a reduction of alpha diversity and shift in microbial composition short after treatment (14 days). However, long-term (13-39 month) impact of azithromycin on gut microbiota composition was not observed. Our results alleviated concerns with respect to the adverse effects of antibiotics treatment on gut microbiota.

**MANUSCRIPT 2**, “Glucose-lowering medication and lifestyle intervention drive the gut microbiota in a similar direction: A randomized clinical trial”, is a study aiming to compare the effect of glucose-lowering medication treatment and lifestyle intervention on gut microbiota for patients with type 2 diabetes (T2D). We found that although both lifestyle intervention and medication treatment shifted the gut microbiota tremendously over a 12-month follow-up, these two therapies did not lead to different changes in taxonomic composition and functional potential of gut microbiota. We concluded that glucose-lowering medication especially metformin drives the gut microbiota of diabetic patients in parallel with the effect of lifestyle intervention.
MANUSCRIPT 3, “A glance into spatial and individual variability of rhizosphere microbiota”, is a study aiming to investigate the rhizosphere microbiota in different sampling scales. At the scale of a single root, we showed that rhizosphere microbiota are highly heterogeneous along root axis in alpha diversity, beta diversity, taxonomic composition, but also in the response to inoculation. Conversely, the traditional sampling scale by homogenizing the entire root system reduced the variance between replicates, giving higher reproducibility at the expense of not fully seeing the influence of inoculation on rhizosphere microbiota.
**Resume**

Bakterier, den første livsform der opstod på jorden, er tilstede i de fleste globale habitater, såsom jord, vand, skyer, varme kilder, og jordens skorpe etc. Af alle mulige habitater er de mikrobielle samfund ved grænsefladerne hos store værtsorganismer, såsom den menneskelige tarm og omkring planterødder, utrolig komplekse økosystemer. Den relativt stabile sameksistens af mikrobiotaer med deres vært antyder den økologiske signifikans af holobionter; enheden bestående af den makroskopiske vært og dens associerede mikrobiota.

Vi udførte tre studier med det formål at undersøge symbionters relevans for værtens helbred og metabolisme. I to studier omhandlende den menneskelige tarm, undersøgte vi hvordan brug af medicin, og forbedret kost og motion påvirker tarmmikrobiotaen. I det tredje studie undersøgte vi forskellige aspekter af rhizosfære-mikrobiotaen hos *Brachypodium distachyon*, såsom skalaen for prøveudtagning (traditionel vs. subskala), lokation på roden (rodsektioner), variationer indenfor og imellem planter, og effekten af inokulation.

Arbejder præsenteret her bygger på tre manuskripter:


**MANUSKRIP 2**, “Glucose-lowering medication and lifestyle intervention drive the gut microbiota in a similar direction: A randomized clinical trial”, er et studie med det formål at undersøge effekten af glukosesænkende medicinering eller livsstils intervention på tarmmikrobiotaen hos patienter med type 2-diabetes. På trods af at både livstilsintervention og metformin behandling ændrede tarmmikrobiotaen enormt over en 12 måneders periode, var der ingen forskel på udviklingen af tarmmikrobiotaen ved disse to behandlinger. Vi
konkluderede at glukosesænkede medicinering, specifikt metformin, ændrede tarmmikrobiotaen hos diabetiske patienter på samme niveau som effekten af livsstilsintervention.

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Abbreviations

COPSAC: Copenhagen Prospective Studies on Asthma in Childhood
OTU: Operational taxonomic unit
T2D: Type 2 diabetes
ISR: Induce systematic resistance
IBD: Inflammatory bowel disease
SCFA: Short-chain fatty acid
VOC: Volatile organic compound
ACC: 1-aminocyclopropane-1-carboxylate
IAA: Indole-3-acetic acid
PGPRs: Plant growth promoting rhizobacteria
RCT: Randomized clinical trial
LPS: Lipopolysaccharide
1 Introduction

Antony van Leeuwenhoek’s discovery of unicellular organisms in 1674 (Corliss, 1975) was the first step leading to the establishment of a new scientific discipline that is microbiology. Nowadays, with the advent of high throughput sequencing technologies and sophisticated analytic techniques, we are able to elucidate complex microbial communities in numerous sites (Costello et al., 2009; Hacquard et al., 2015).

Human gut is a digestion tract starting from mouth to anus in which food is digested and nutrient is absorbed. The two major sections of the gut are small intestine and large intestine, which has a combined length of around 8 meters (Hounnou et al., 2002). Gut is estimated to have a surface area of 32 m² mainly due to the millions of fingerlike villi in the small intestine (Helander and Fändriks, 2014). Facilitated by such huge surface area, gut harbors a reservoir of microbes that are in a comparable number to our own cells (Sender et al., 2016), which makes it one of the most complex niches compared to other body sites (Costello et al., 2012; Martin, 2012; Mortensen et al., 2016).

Plant roots are overpopulated by a variety of microbes either residing inside the root tissue and on the root surface or living in a narrow zone surrounding roots called rhizosphere. Consistent with the function of microvilli and folds in gut, root hairs massively extend the surface area of roots (Grierson and Schiefelbein, 2002). Rhizosphere microbiota are mainly recruited from the surrounding soil which is considered as one of the largest reservoir of microbial diversity known so far (Torsvik et al., 2002) and play an important role in the relationships between diversity and ecosystem functioning. Rhizosphere microbiota have short turnover time and large population size which makes rhizosphere a special hotspot possibly having the highest total turnover of cells per year compared to other habitats (Reinhold-Hurek et al., 2015). With the context of large microbial population surrounding roots and its potential significance to the fitness of plant host, rhizosphere was vividly seen as an “inside out gut” (Ramírez-Puebla et al., 2013).

1.1 Gut microbiota

Human body surfaces are home to unique microbial communities, of which the gut microbial community is the most thoroughly studied. The gut is a digestive tract with large surface area and harbors $3.8 \times 10^{13}$ microbes reaching overall biomass of 0.2 kg (Sender et al., 2016). Compared to the stomach where only 10 microbial cells reside per ml (Verdu et
al., 2015), the majority of gut microbiota are residing in the colon (also called large intestine) where densities approach $10^{11}-10^{12}$ cells/ml, which makes colon the most densely colonized human body habitat (Whitman et al., 1998). Collectively, over 1000 species reside in our gut and contain 100 times more genes than the human genome (Eckburg et al., 2005; Gill et al., 2006). Due to the limited oxygen availability, the majority of gut microbiota are strict anaerobes, which dominate the facultative anaerobes and aerobes by two or three orders of magnitude (Sekirov et al., 2010).

1.1.1 Gut microbiota in health and disease

The gut microbiota are a reservoir of diverse microbes that play an important role in the well-being of their host through immune (Round and Mazmanian, 2009) and metabolic functions (Tremaroli and Bäckhed, 2012) (Fig. 1), therefore sometimes gut is considered as our “forgotten organ” (O’Hara and Shanahan, 2006). In terms of gut microbial composition, Bacteroidetes and Firmicutes are the two dominant phyla in adults which makes up over 90% of identified phylogenetic types and followed up by Proteobacteria, Verrucomicrobia, Actinobacteria etc. The abundance ratio between Bacteroidetes and Firmicutes has been linked to the homeostasis of human metabolism. The reduction of Bacteroidetes and increase of Firmicutes has been associated with obesity both in mice and human (Ley et al., 2005; Ley et al., 2006). Proteobacteria are mostly facultative anaerobic and known for their indication of gut microbiota dysbiosis (Shin et al., 2015) via dysbiotic expansion through aerobic respiration since intestinal inflammation or antibiotic treatment increases epithelial oxygenation (Litvak et al., 2017). Verrucomicrobia are occasionally observed and might indicate a healthy status (Fujio-Vejar et al., 2017), especially the bacterium *Akkermansia muciniphila* has been associated with many metabolic disorders, such as overweight, obesity, T2D (Derrien et al., 2017). Actinobacteria only represent a small percentage of the total abundance but are involved in numerous physiological functions (Binda et al., 2018). Actinobacteria, especially *Bifidobacterium* and *Lactobacillus*, are used/considered as probiotics which provide health benefits. Although there are diverse members of gut microbiota, evidences have shown the presence of a core microbiota and clusters of robust community types (called enterotypes) of gut microbiota regardless of age, gender or body mass index (Turnbaugh et al., 2009; Arumugam et al., 2011; Burke et al., 2011) which boosted the investigation of gut microbiota in health and disease (Clemente et al., 2012).
Commensal gut microbiota benefits host health in multiple ways. The gut microbiota help degrade a number of tough polysaccharides, such as plant-derived pectin, cellulose, hemicellulose, and resistant starches to recover calories (Bäckhed et al., 2005). Microbiota residing in the colon produce short-chain fatty acids (SCFAs) via anaerobic fermentation of dietary fibers (Cummings et al., 1987). SCFAs are crucial for mammalian energy metabolisms, such as butyrate which is an important energy source and regulatory molecule for epithelial cells (coloncytes) (Christl et al., 1996; den Besten et al., 2013). The gut microbiota supply vitamins to their host, which can only be provided exogenously (LeBlanc et al., 2013). By competing for nutritional substrates and ecological niches, gut microbiota members protect against pathogens invasion (Kamada et al., 2013). Furthermore, evidence was reported that gut microbiota are involved in the modulation of host immune responses possibly via induction of specialized T cells known as regulatory T (T_{Reg}) cells to limit inflammation and disease (Round and Mazmanian, 2009). The
significance of microbial function in mammalian development is evident, as germ-free mice are both physiologically and immunologically aberrant and such phenotype can be rescued via early introduction of commensal bacteria (Atarashi et al., 2011). Quigley et al. (2013) and F. Fava et al. (Fava et al., 2018) have presented detailed summaries of how gut microbiota are beneficial to human.

Growing evidence shows that dysbiosis of gut microbiota is related to both intestinal and extra-intestinal disorders. Intestinal disorders include inflammatory bowel disease, coeliac disease, and irritable bowel syndrome, while extra-intestinal disorders include asthma, obesity and type 2 diabetes (T2D) (Carding et al., 2015). I will discuss asthma and T2D in more details as they are related to Manuscript 1 and Manuscript 2 in this thesis, respectively.

Asthma is caused by a complex combination of genetic and environmental factors (Martinez, 2007). Although airway microbiota has been naturally the focus of asthma studies (Fujimura and Lynch, 2015b), growing literature has shown gut microbiota, especially in the early life, is linked to the pathogenesis of asthma. Many early life exposures have been associated with increased risk of later allergic disease, such as antibiotics exposure (Johnson et al., 2005), cesarean birth (Renz-Polster et al., 2005), formula feeding (Friedman and Zeiger, 2005), lack of older siblings (Karmaus and Botezan, 2002), maternal consumption of antimicrobials during pregnancy (Stensballe et al., 2013) and lack of exposure to pets (Ownby et al., 2002). Antibiotics administration reduces gut microbiota diversity massively (Wei et al., 2018), while cesarean section delays gut microbial maturation, including Bacteroidetes colonization and reduces Th1 responses in infants (Jakobsson et al., 2014). Formula feeding infants are linked to decreased abundance of commensal Bifidobacteria (Balmer and Wharton, 1989). The number of older siblings is positively correlated with bacterial diversity (especially of the phyla Firmicutes and Bacteroidetes) and richness (Laursen et al., 2015). Household pets were shown to increase the abundance of genera Ruminococcus and Oscillospira and have been negatively associated with childhood atopy and obesity (Tun et al., 2017). Asthma at school age is preceded by reduced gut microbial maturity in early life (Bisgaard et al., 2011; Abrahamsson et al., 2014) and eight genera of gut microbes in 1-year old children were associated with asthma at age 5 in children born to asthmatic mothers (Stokholm et al., 2018). All the above studies demonstrate the association of gut microbiota in early life, a critical period of microbiological and immunological development, with the risk of
asthma development. One explanation behind the prevalence of allergic disease is the hygiene hypothesis which was first suggested by David P. Strachan (1989). He showed that the hay fever at both 11 and 23 years of age was inversely correlated with the number of children in the household at 11 years of age. The hygiene hypothesis was thereafter revisited by Wold (1998) who highlighted the importance of gut microbiota. However, to date, the hygiene hypothesis is still evolving and a unifying concept has not emerged yet (Guarner et al., 2006; Schaub et al., 2006). Ecologically, development of gut microbiota in early life resembles the “primary succession” where pioneer species frequently determine the following ecosystem conditions (Fujimura and Lynch, 2015a), therefore abnormal gut microbiota in early life may result in the loss of key commensal species for proper physiological and immune development.

The prevalence of type 2 diabetes (T2D) has rapidly risen over the past decade(s) and become a global health issue (Dabelea et al., 2014; World Health Organization, 2016). T2D is a complex disorder resulting from both genetic and environmental components, of which overweight and obesity are the strongest risk factors (World Health Organization, 2016). Interestingly, evidence has shown that gut microbiota are related to such metabolic syndromes. Over the past decade, we progressed toward a clear understanding of the link between gut microbiota and overweight/obese. The gut microbiota of obese individuals represent an “obese microbiota phenotype” characterized by a reduced abundance of Bacteroidetes and increased prevalence of Firmicutes (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2006). It has been further proven in mice that obese-associated microbiota harbors greater energy harvesting abilities, driven by the phylum Firmicutes and its obesity-linked feature, which could be transferred between individuals (Turnbaugh et al., 2006). Besides, short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, were all elevated in fecal samples from obese humans and mice (Turnbaugh et al., 2006; Fava et al., 2013). T2D is characterized by insulin resistance in the peripheral tissues and a relative deficiency of insulin secretion from the beta cell in the pancreas (Zander et al., 2002) and is usually preceded and accompanied by obesity. Studies have shown the possible significance of the composition of gut microbiota in T2D. Qin et al. (2012) showed a lower abundance of butyrate-producing bacteria in diabetic subjects compared to no-diabetic controls and increased the abundance of opportunistic pathogens. Besides, diabetic subjects showed higher energy harvesting ability at the pathway level, which is consistent with Turnbaugh’s argument (Turnbaugh et al., 2006). A comparable study by Karlsson (2013) found increased abundances of four Lactobacillus species and
decreases in the abundance of five Clostridium species. Roseburia and Faecalibacterium prausnitzii, identified as the most discriminant bacteria for T2D, are well-known butyrate producers, which again supports the concept that “obese microbiota phenotype” residing in obese individuals harbors increased capacity for energy harvest. Obesity and T2D are not always disentangled from each other since they are usually pathophysiological overlapped. In terms of gut microbiota, a road from obesity to diabetes could be: High fat induces obesity accompanied with alteration of gut microbiota; This leads to gut permeability via disturbance of tight junction proteins (Zonula Occludens-1 and Occludin); These phenomena increase plasma lipopolysaccharide (LPS) level, termed metabolic endotoxemia, and initiate the development of low-grade inflammation and insulin resistance via innate immune system. A detailed summary that linking gut microbiota to obesity and type 2 diabetes can be found in (Cani et al., 2012)

1.1.2 Factors driving gut microbiota

Numerous factors influence the homeostasis of gut microbiota, they can be categorized into host factors (such as pH, bile acids, physical exercise), non-host factors (such as antibiotics, diet) and bacterial factors (such as adhesion capacity, enzymes) (Goossens et al., 2003; Penders et al., 2014). This section will focus on medication, diet, and physical exercise.

Fig. 2. Factors shaping gut microbiota and the effects of dysbiosis on host health. The microbial composition of gut microbiota can be influenced by factors, including antibiotics, lifestyle, diet, which are shown, and many others. Adapted and modified from (Sommer and Bäckhed, 2013).
1.1.2.1 Antibiotics

Numerous evidences have shown antibiotics have a profound impact on gut microbiota. A reduced microbial richness, diversity, and evenness in the gut are often linked to the antibiotics administration (Table 1). Besides, antibiotics can also shift microbial composition even at phylum level, such as increase of Proteobacteria (Fouhy et al., 2012; Korpela et al., 2016) and Firmicutes (Panda et al., 2014), decline of Actinobacteria (Jakobsson et al., 2010; Fouhy et al., 2012; Korpela et al., 2016; Wei et al., 2018), Bacteroidetes (Jakobsson et al., 2010; Panda et al., 2014), and Verrucomicrobia (Parker et al., 2017). However, conflicting results were also reported, such as decrease of Proteobacteria (Parker et al., 2017) (against Korpela’s work) which might due to the difference of baseline microbial composition and antibiotic resistance genes. Besides, different antibiotics can lead to a distinct shift in microbial composition (Korpela et al., 2016), which highlights the antibiotic-specific effect on gut microbiota.

In addition to short-term effect, studies have shown the potential long-term effect of antibiotics on gut microbiota. For instance, some taxa failed to recover to the initial state 6 months after treatment (Dethlefsen and Relman, 2011), the microbial diversity and richness (Zaura et al., 2015; Korpela et al., 2016) as well as maturity remained reduced (Korpela et al., 2016) after 12 months, with persisting high level of resistance genes years after (Jernberg et al., 2007; Jakobsson et al., 2010; Yassour et al., 2016). However, there has been disagreement among studies. Korpela et al. (2016) showed that 6-12 months after treatment, the macrolides resistance genes declined to the baseline level. Besides, the MANUSCRIPT 1 showed that the long-term azithromycin effect on the gut microbiota composition in children is absent. Such discrepancy could be derived from different antibiotics used, population characteristics, and investigation methods.

In addition to the compositional alteration, antibiotics also affect gene expression, protein activity and overall metabolism of gut microbiota (Francino, 2016). For instance, β-lactam results in elevated and less balanced sugar metabolism, which is similar to that observed in obese individuals (Hernández et al., 2013). A thorough experiment of gut microbiota treated with different antibiotics ex vivo has shown a number of impacts on gut microbiota caused by antibiotics treatment, such as increased proportions of damaged cells (because of disturbance on cell-wall synthesis), elevated expression of genetic information processing genes (transcription and translation), induction of gene expression for drug resistance and metabolism (Maurice et al., 2013).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects/Study type</th>
<th>Antibiotics</th>
<th>short-term (&lt;6 months)</th>
<th>long-term (&gt; 6 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jernberg et al. (2007)</td>
<td>4 adults; 4 adults, control/ob</td>
<td>clindamycin</td>
<td>Day 0, 7, 21 and m 3, 6; decline of colonial Bacteroides, increase of resistant clone types</td>
<td>9, 12, 18 and 24 m; decline of colonial Bacteroides, increase of resistant clone types</td>
</tr>
<tr>
<td>Dethlefsen et al. (2008)</td>
<td>3 adults with urinary tract infection/ob</td>
<td>500 mg ciprofloxacin BID for 5 days</td>
<td>3-4 days; Abx significantly reduced richness in 2 out of 3 patients, Shannon’s diversity in all 3 patients</td>
<td>180 days; incomplete (some participants, 10 m)</td>
</tr>
<tr>
<td>Jakobsson et al. (2010)</td>
<td>3 adults with dyspeptic disorder: No Abx; 3 adults with gastric/duodenal ulcers: Abx/ob</td>
<td>Metronidazole 400 mg, clarithromycin 250 mg BID for 7 days</td>
<td>8-13 days; Abx reduced Shannon’s diversity index.</td>
<td>1 and 4 years; partially recovered to initial state</td>
</tr>
<tr>
<td>Dethlefsen et al. (2011)</td>
<td>5 healthy adults/ob</td>
<td>Ciprofloxacin</td>
<td>3-5 days; Abx reduced richness and Shannon’s diversity index.</td>
<td>6 m; several taxa failed to recover</td>
</tr>
<tr>
<td>Fouhy et al. (2012)</td>
<td>9 untreated/9 treated infants (within 48 hours of birth)/ob</td>
<td>Parenteral penicillin and gentamicin</td>
<td>4, 8 weeks; Abx reduced Shannon’s diversity index and Chao1 index.</td>
<td>No data</td>
</tr>
<tr>
<td>Pérez-Cobas et al. (2013)</td>
<td>1 patient with infected cardiac pacemaker/ob</td>
<td>Ampicillin/sulbactam and cefazolin</td>
<td>3, 6, 11, 14 and 40 days; Abx reduced Shannon’s diversity index and Chao1 index.</td>
<td>No data</td>
</tr>
<tr>
<td>Dardas et al. (2014)</td>
<td>27 preterm infants (&lt;32 weeks); 15 received 2 days of Abx &amp; 12 received 7-10 days of Abx/ob</td>
<td>Ampicillin, gentamicin</td>
<td>7-10 days of Abx significantly reduced Shannon’s diversity compared to 2 days of Abx; no statistical significance in richness</td>
<td>No data</td>
</tr>
<tr>
<td>Panda et al. (2014)</td>
<td>21 hospitalized adult patients (18-80 yo)/ob</td>
<td>Amoxicillin-clavulanate, levofloxacin, metronidazole, ceftriaxone, azithromycin, ciprofloxacin, piperacillin/tazobactam</td>
<td>7 days; Abx decreased Chao1 index, shifted microbial composition</td>
<td>No data</td>
</tr>
<tr>
<td>Arat et al. (2015)</td>
<td>62 healthy adults (18-65 yo)/RCT</td>
<td>GSK1322322 (GlaxoSmithKline)</td>
<td>6 days; Oral/IV Abx reduced Chao1 index.</td>
<td>No data</td>
</tr>
<tr>
<td>Rashid et al. (2015)</td>
<td>30 healthy adults (18 to 45 yo)/ob</td>
<td>Ciprofloxacin 400 mg BID for 10 days, clindamycin 150 mg QID for 10 days</td>
<td>11 days and 1, 2, 4 m; Abx significantly reduced taxa richness</td>
<td>12 m; Changes in microbial composition</td>
</tr>
<tr>
<td>Zaura et al. (Zaura et al., 2015)</td>
<td>30 healthy adult volunteers in Sweden and 44 volunteers in the United Kingdom/RCT</td>
<td>Ciprofloxacin, clindamycin, amoxicillin, minocycline</td>
<td>1 week and 1, 2, 4 m; Abx significantly reduced taxa richness up to 2 m after treatment. Based on Bray-Curtis dissimilarity, statistically significant up to 4 m</td>
<td>12 m, reduced Shannon diversity (ciprofloxacin group)</td>
</tr>
<tr>
<td>Bokulich et al. (2016)</td>
<td>43 infants from birth to 3 years of life/ob</td>
<td>Nitrofurantoin, penicillin, amoxicillin, cephalexin, azithromycin</td>
<td>0-12 m; phylogenetic diversity and richness differed between groups. Antibiotics delayed gut microbiota maturation between 6-12 m</td>
<td>2-3 years; no difference</td>
</tr>
<tr>
<td>Korpela et al. (2016)</td>
<td>142 children (2-7 yo)/ob</td>
<td>Amoxicillin with or without clavulanic acid, penicillin V, azithromycin, clarithromycin, sulphonamide-trimethoprim</td>
<td>6 m; Penicillins and macrolides both reduced richness and relative maturity. Macrolides increased macrolide resistance genes level</td>
<td>Penicillins reduced richness at 12 m; macrolides reduced richness and maturity at 12 and 24 m; macrolide resistance genes reached to baseline level at 12 m.</td>
</tr>
<tr>
<td>Yassour et al. (2016)</td>
<td>39 infants receiving none or &gt; 9 Abx courses in the first 3 years of life/ob</td>
<td>Aminoglycosides, cephalexins, macrolides, penicillins, sulphonamides</td>
<td>variable time interval; Abx reduced Chao 1 index, microbial communities stability, increased resistance genes</td>
<td>6-30 m; episomal encoded resistance genes.</td>
</tr>
<tr>
<td>Doan et al. (2017a)</td>
<td>80 children in Niger, 40 placebo, 40 azithromycin/RCT</td>
<td>Azithromycin</td>
<td>5 days; Abx reduced Shannon and Simpson index and shifted microbial composition</td>
<td>No data</td>
</tr>
</tbody>
</table>
Table 1. Studies on the effects of antibiotics on the gut microbiome of adults and children.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects/Study type</th>
<th>Antibiotics</th>
<th>short-term (&lt;6 months)</th>
<th>long-term (&gt;6 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parker et al. (2017)</td>
<td>120 children in India, 60 placebo, 60 azithromycin / RCT</td>
<td>Azithromycin</td>
<td>12 days; Abx reduced the Chao 1 index and shifted microbial composition</td>
<td>No data</td>
</tr>
<tr>
<td>Wei et al. (2018)</td>
<td>100 children in Denmark, 29 placebo, 30 azithromycin / RCT</td>
<td>Azithromycin</td>
<td>14 days; Abx reduced richness and Shannon index and shifted microbial composition</td>
<td>13-39 m; Abx did not have long-term effect</td>
</tr>
</tbody>
</table>

Abbreviations: Abx, antibiotics; BID, twice daily; QID, four times a day; yo, years old; ob, observational; m, month. RCT, randomized clinical trial. We arbitrarily consider 6 months as the threshold to differentiate short- and long-term of antibiotic effect on the gut microbiota. The table is adapted and modified from (Doan et al., 2017a).

1.1.2.2 Diet

Diet is a key determinant for gut microbiota composition. Dietary pattern (western or vegetarian diet), specific foods (fruits, grain or vegetables) and foods constituents (fiber, protein or fat) all can lead to the change of gut microbiota (Graf et al., 2015). Western diet is usually characterized by a high amount of fat and low in fiber. A number of studies have investigated the influence of western diet on gut microbiota by comparing individuals between less developed and developed countries. For instance, Malawian infants were shown to harbor higher level of *Bifidobacteria* in their gut than Finnish (Grześkowiak et al., 2012). Both children and adults in United States (US) were different from populations in Amazonas of Venezuela and rural Malawi in terms of gut microbiota overall structure and US adults had the least diverse gut microbiota compared to other two countries (Yatsunenko et al., 2012). The gut microbiota of Africans from a rural village of Burkina Faso represented an enrichment of Bacteroidetes, depletion of Firmicutes and more SCFAs than Europeans (De Filippo et al., 2010). More abundant of butyrate-producing groups were in the fecal samples from native Africans than African Americans (Ou et al., 2013). Tanzanians were higher in microbial richness and diversity than urban Italian (Schnorr et al., 2014).

In mice, high fat diet has been shown to reduce genes coding for tight junction proteins, causing increased intestinal permeability and thereby leading to metabolic disorders (Cani et al., 2008), such as obesity and diabetes, as discussed above. In contrast, dietary fiber would stimulate the growth and activity of butyrate-producing bacteria, benefiting our health both locally and systemically (Graf et al., 2015). For a detailed summary of the influence of foods on the gut microbiota, please refer to Graf et al. (2015).
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1.1.2.3 Physical activity

Physical activity, especially well-planned and structured physical exercise, is beneficial to our health in many ways and its adoption and maintenance are critical for overall health in individuals with obesity and prediabetes (Colberg et al., 2016). A series of studies demonstrated the benefits of exercise, such as improvement in age-related cognitive impairment (Bherer et al., 2013), prevention of colon cancer (Robshahm et al., 2013), treatment of T2D (Johansen et al., 2017), irritable bowel syndrome (Johannesson et al., 2011) and depression (Agudelo et al., 2014; Harkin, 2014). Given the role of gut microbiota in health and disease, the obtained physiological fitness might partially be the function of gut microbiota modulation (Cerdá et al., 2016). It should be noted that mild-to-moderate intensity and regular exercise is protective, but excessive, intensive exercise could lead to intestinal injury which is correlated with transiently increased small intestinal permeability (O’Sullivan et al., 2015). In literature, numerous studies have shown that lifestyle change or physical activity reduce the risk or incidence of disease, but studies regarding how the gut microbiota are involved in such processes remain relatively scarce and the work has generally been done using murine models.

Exercise in rats induced a significant increase of *Lactobacillus*, *Bifidobacterium* and *Blautia cocoides-Eubacterium rectale* group (in which many are butyrate-producing species) (Queipo-Ortuño et al., 2013). In mice, exercise reduces the abundance of *Streptococcus* massively (Kang et al., 2014) and increases distal gut microbial diversity and metabolic capacity (Denou et al., 2016). A comparable study in mice showed exercise could reduce intestinal inflammation and prevent high-fat induced morphological changes in proximal and distal gut. The species *Faecalibacterium prausnitzii*, an important butyrate-producer, was only observed in exercised mice (Campbell et al., 2016). Athletes, compared to more sedentary subjects, represent increased gut microbial diversity and enhanced production of amino acids, SCFA (propionate and butyrate), and overall health (Clarke et al., 2014; Barton et al., 2017).

Accumulating literature has shown the positive effects of physical exercise on the mental problem, such as anxiety, stress, and depression (Mikkelsen et al., 2017), during which gut microbiota might act as a mediator for the effect of exercise on the brain (Yuan et al., 2015).

1.2 Root microbiota
Plants are populated by a variety of microorganisms in different sites termed “sphere” (Johansson et al., 2004; Nelson, 2004; Bulgarelli et al., 2012; Vorholt, 2012). Compared to other “spheres”, the hidden world below ground represents an even more important role for host health given root microbiota serve additional functions via the nutrients acquisition from soil supporting plant growth (Bulgarelli et al., 2013). These root microbiota appear to be symptomless in many situations and represent as symbiosis ranging from commensalistic to mutualistic interactions with their hosts. To the benefits of plants, root microbiota can provide nutrient acquisition, pathogen protection services to their host and extend the capacity of plants to adapt to the environment. Collectively, all these root-associated microorganisms are referred to as plants’ other genome.

The microhabitats provided by roots at the soil-root interface can be divided into three zones, namely rhizosphere, rhizoplane, endorhizosphere. Rhizoplane is the root surface where bacteria reside including root epidermis and the outer cortex. Soil particles and microorganisms can adhere to this area and possibly form biofilms (Bogino et al., 2013). The rhizoplane microbiota was shown to harbor growth-improving abilities (Siddiqa et al., 2016). Endorhizosphere is the inner root tissue compartment including rhizodermis, cortex, and stele. Specialized features have selected for bacteria to colonize within root tissue such as cell wall degrading enzymes, twitching motility, lipopolysaccharides, flagella, and pilus (Duijff et al., 1997; Dörr et al., 1998; Lodewyckx et al., 2002; Böhm et al., 2007). For instance, the well-known rhizobia have evolved specific mechanisms to enter the root system (reviewed in Hardoim et al., 2008). For more details about endophytes, please refer to (Hardoim et al., 2008). My following discussion will mainly focus on the rhizosphere. The root-associated microbiota primarily includes bacteria, fungi, and archaea. Compared to bacteria, Archaea is much less prominent and does not appear to associate tightly with plants (Reinhold-Hurek and Hurek, 2011), therefore is not included in my discussion. Fungi are an important plant root symbioses and occur in different forms referred to as mycorrhiza such as arbuscular mycorrhiza that is formed by 70~90% of land plant species. However, fungi are not relevant to this thesis and will not be discussed in detail. More details can be found in (Parniske, 2008). My following introduction will mainly focus on bacteria.

1.2.1 Root microbiota assembly

Soil is the largest reservoir of microbial diversity ever known (Torsvik et al., 2002). Root microbiota are recruited from this reservoir since the surrounding soil is likely the starting
inoculum for roots, although there exist seed-transmitted endophytes (Truyens et al., 2015). Reinhold et al. (2015) have provided a three-step enrichment model to describe the assembling of root microbiota (Fig. 3). It is as such: First, microbes are refined in the rhizosphere soil, the soil attached to and affected by plant roots, by the factor gradients such as carbon sources, pH, oxygen etc. Second, on the rhizoplane, root surface where microbes reside, a more specialized community is enriched where close interactions between microbes and host occur. Plant genotype has a strong impact on the selection of microbes. Third, endorhizosphere, inner root tissue inhabited by microbes, where only bacteria having specific features can reside there.

**Fig. 3.** A three-step model for microorganisms colonizing different root microhabitats. Three numbers refer to three enrichment steps accordingly. From the outside (soil) to inside (root tissue), bacterial diversity decreases and the influence of plant genotype increases. Bulk soil (without roots) represent as the reservoir of microbes which is influenced by a series of factors, such as soil type and environmental factors.
In addition to the recruitment of microbes from the surrounding local soil, seed-transmitted inoculation is an additional source (Truyens et al., 2015). Studies have shown the significance of seed endophytes for the health benefits of rice (Hardoim et al., 2012), maize (Johnston-Monje and Raizada, 2011; Johnston-Monje et al., 2016), cactus (Puente et al., 2009), switchgrass (Gagne-Bourgue et al., 2013), tomato (Xu et al., 2014), and more (Truyens et al., 2015). Seed endophytes can move within the plant tissue, even able to spread out into the rhizosphere, and are guaranteed to be the part of the starting inoculation package that will seed the roots. More details regarding the potential health benefits provided by seed endophytes can be found in (Truyens et al., 2015).

1.2.2 Rhizosphere

Rhizosphere is a narrow zone surrounding and influenced by plant roots and represents as one of the most complex and dynamic ecosystems on Earth (Philippot et al., 2013). Microbes living in rhizosphere can utilize the large amount of rhizodeposits (e.g. border cells, exudates, mucilage) released by the plant, therefore flourish into a community in which elevated microbial activities and biomass were observed compared to bulk soil (Norton and Firestone, 1991; Söderberg and Bååth, 1998; Marschner et al., 2011; Marschner et al., 2012; Ofek et al., 2014).

1.2.2.1 Taxonomic and functional composition

Although the composition of rhizosphere microbiota varies according to a series of factors such as root zone, soil type, plant species, stress and disease, and plant phonological phase (Lagos et al., 2015), dominant bacterial phyla are Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Acidobacteria (Kawasaki et al., 2016). Proteobacteria is commonly being the most dominant phylum among different plants (Mendes et al., 2013; Philippot et al., 2013) due to their ability to rapidly respond to labile carbon sources, fast growth rate, and generally considered as r-strategist (Cleveland et al., 2007; Fierer et al., 2007; Peiffer et al., 2013). It has been shown that an order of Proteobacteria, Burkholderiales, assimilate carbon rapidly by providing $^{13}$CO$_2$ to plants (Vandenkoornhuyse et al., 2007). Similar as Proteobacteria, Firmicutes (at genus level it is Bacillus) also harbor fast response to labile C compounds (Cleveland et al., 2007). Such ability is beneficial for Proteobacteria and Firmicutes for utilizing carbon sources released from roots in the dynamic rhizosphere. Next-generation sequencing enables to characterize rhizosphere microbiota with considerable resolution. Accompanied with such techniques,
rhizosphere microbiota are often reported to be lower in richness than bulk soil, which is reasonable considering the assembling process of root-associated microbiota, where root exudates and plant genotype are acting as filters selecting for a highly specialized microbiota (Reinhold-Hurek et al., 2015).

In addition to taxonomic composition of rhizosphere microbiota, it is also important to investigate the microbial functions and pathways displayed in rhizosphere. Recently with the advances of sequencing techniques such as metagenomics, metatranscriptomics, and metaproteogenomics, studies have identified the gene transcripts, proteins, or metabolites of rhizosphere microbiota in many plants (Wang et al., 2011; Wu et al., 2011; Knief et al., 2012; Turner et al., 2013). A study using RNA-based metatranscriptomics compared the microbiota in rhizosphere soil and bulk soil across wheat, oat, and pea (Turner et al., 2013). It showed that the selection of rhizosphere microbiota by plants is partially based on the metabolic capabilities of microbes. For instance, cellulolytic bacteria such as phylum Fibrobacteres and genus Cellvibrio were enriched in wheat and oat because of the presence of plant cell-wall material in the rhizosphere. These studies provide an inference of physiological traits of rhizosphere microbial communities. For more details, please look at (Mendes et al., 2013).

1.2.2.2 Spatial variation of rhizosphere microbiota along root axis

Numerous studies have thoroughly addressed and well characterized the heterogeneity between rhizosphere and bulk soil (soil without roots). In contrast, the heterogeneity along root axis is much less investigated. The heterogeneity of rhizosphere microbiota along root axis is driven by the variation of the physical, chemical and biological characteristics. Carbon often represents as the most influential factor limiting the growth and activity of soil microorganisms (Demoling et al., 2007). Hence, root exudates stand as the key in shaping the rhizosphere microbial communities. Therefore, the variation of root exudates in amount and composition reflects the variation of rhizosphere microbiota. Rhizodeposits include a variety of substances, such as sugars, amino acids, organic acids etc., that are originated from detached border cells, mucilages, volatiles, and exudates released from damaged and intact cells (Dennis et al., 2010). Root cap is the place where a large amount of mucilages are released accounting for 2~12% of the total rhizodeposition (Dennis et al., 2010). Border cells detached and lysed from root cap represent 10% of all carbon released by roots (Iijima et al., 2000). Behind root cap, the meristematic zone and elongation zone also release a vast amount of root exudates (Dennis et al., 2010; Doan et al., 2017b).
Therefore, a high microbial density is expected to occur in the vicinity of root tip and has been observed in several studies (Watt et al., 2003; Watt et al., 2006; DeAngelis et al., 2009). Accompanied by spatial variation of root exudates and microbial metabolism, the heterogeneity of pH and enzymatic activities along root axis are also important to consider. The decomposition of soil organic matter and root exudates relies on enzymes and a study in maize revealed that enzyme activities were decreasing farther away from the root tips (Razavi et al., 2016). In a similar pattern, the soil pH surrounding root tip is the lowest in lupin and soft-rush plants (Rudolph et al., 2013). Oxygen is fundamental for aquatic plants such as rice, as the majority of them living in flooded and waterlogged soils. To prevent harmful reduced/toxic chemicals such as H$_2$S and Fe$^{2+}$, plants are able to leak an O$_2$ layer around roots. It has been shown that intense leakage of O$_2$ is at root tips (Larsen et al., 2015), mainly due to the effective barrier to radial oxygen loss on the older part of roots and the presence of an effective gas-transport system in the plants (Jensen et al., 2005). Santner (2015) has summarized articles using different imaging methods to investigate the spatial variation of soil elements including O$_2$, CO$_2$, pH, redox conditions and nutrients, and iron.

The spatial distribution of rhizosphere microbes has been investigated in a range of plants such as oat (DeAngelis et al., 2009), wheat (Semenov et al., 1999; Watt et al., 2003; Watt et al., 2006), tomato (Chin-A-Woeng et al., 1997; Gamalero et al., 2004) and cucumber (Folman et al., 2001). Besides, the variation driven by variable amount of root exudates released along root axis (root zones), root types (primary and secondary) (Kawasaki et al., 2016) and the movement of roots while growing through the soil also might contribute to the heterogeneity along root axis (Philippot et al., 2013).

**1.2.3 Determinants of root/rhizosphere microbiota**

Soil is the reservoir of numerous microbes from which roots select their microbiota, therefore, soil type (sand, loam, clay etc.) likely stands as the most influential factor shaping the composition of microbial communities due to a number of factors, such as fertility, porosity, organic matter to minerals ratio, water holding capacities etc. The strong influences of soil type have been investigated in many studies (Bulgarelli et al., 2012; Lundberg et al., 2012; Schreiter et al., 2014). However, for strawberry, plant genotype seems to exert a stronger influence on microbial composition than soil type (Weinert et al., 2011).
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Soil pH is another important driver of soil microbial communities. A study investigated the biogeography of bacterial communities in a continental scale across North and South America showed that more than 50% of variation in diversity or richness could be explained by soil pH (Fierer and Jackson, 2006). A comparable study conducted in a large region of France showed that 43–85% of variation in community abundance is attributed to soil pH (Bru et al., 2011).

Under identical living conditions and soils, the influence of plant genotype can be detected. A series of plants including grass (Kuske et al., 2002), tomato (Weinert et al., 2011), maize (Aira et al., 2010; Peiffer et al., 2013), wheat (Rengel et al., 1996), barley (Bulgarelli et al., 2015), Arabidopsis (Micallef et al., 2009) and more (Ofek et al., 2014), have been shown that the structure of microbial community in the rhizosphere differs among different species even though when soil condition is controlled. The difference of root microbial communities driven by genotype is likely due to the difference of various root exudates which are synthesized, released by roots and are under the plant genetic control (Micallef et al., 2009).

It is well-recognized that the composition of rhizosphere communities is variable during plant development stages, namely seeding, vegetative, bolting and flowering, which has been observed in a variety of plants such as Arabidopsis, maize, pea, and wheat (Baudoin et al., 2002; Houlden et al., 2008; Lundberg et al., 2012; Chaparro et al., 2014). In addition to taxonomic composition, a shift in rhizosphere microbial functions over development stages was also observed, such as enhanced root secretion of defense-related protein during flowering time (De-la-Pena et al., 2010) and higher nitrogen demand at later development stages which might facilitate the occurrence of symbiosis between rhizobia and legumes (Zahran, 1999; Malagoli et al., 2004). Again, root exudates might play an important role in the developmental stage-specific microbiota (BADRI and VIVANCO, 2009).

Other factors including redox (Schmidt et al., 2011), drought (Fitzpatrick et al., 2018), heavy metal (DalCorso et al., 2013), and agricultural practices (Upchurch et al., 2008; Mendes et al., 2011) also play a role in shaping root/soil microbial communities.

1.2.4 Health benefits from root/rhizosphere microbiota

To survive in the rhizosphere, bacteria are requested to harbor a variety of traits, such as motility, chemotaxis, attachment, stress resistance and more (Bulgarelli et al., 2013). Once the rhizosphere is colonized, the microbial communities can benefit the plant growth in different ways. I will discuss some of them in the following.
1.2.4.1 Biological nitrogen fixation

Nitrogen fixation is a process by which the atmospheric nitrogen \((N_2)\) converted into ammonia \((NH_3)\). In biology, such process is exclusively carried out among bacteria and archaea instead of eukaryotes, such as the well-know *Rhizobium* representatives which are able to establish inside root nodules and exchange fixed nitrogen for photosynthates (Dixon and Kahn, 2004). These microorganisms utilizing nitrogenases to catalyze the conversion between \(N_2\) and \(NH_3\). Generally, nitrogen is considered one of the limiting nutrients in plant growth. It has been estimated that biological nitrogen fixation from legumes and other nitrogen-fixing microorganisms accounting for 20% of global nitrogen input of cropland (Cassman et al., 2002).

1.2.4.2 Phosphorus solubilization

In soil, only less than 5% of the phosphorus content is bioavailable to plants. But many soil microorganisms are capable to solubilize phosphorus, such as representatives from genera *Bacillus*, *Pseudomonas*, or *Penicillium*. They can liberate phosphates from inorganic and organic phosphorus and related genes have been identified (Rodríguez et al., 2006). Besides, arbuscular mycorrhizae also contribute to the phosphorus solubilization. More details can be found in (Bulgarelli et al., 2013).

1.2.4.3 Iron

Iron \((Fe^{3+})\) is low in availability to plants due to low solubility. Siderophores have a high affinity for iron and serve to transport iron across cell membranes (Hider and Kong, 2010). Rhizosphere microbiota can facilitate the uptake of iron by the production of siderophores (Andrews et al., 2003). Microbes might also protect against fungal pathogenic infection through competing for iron *via* the production of siderophores (Duijff et al., 1999).

1.2.4.4 Phytohormone biosynthesis and interference

Phytohormones are a group of structurally unrelated small molecules, low in concentration, functioning as signal molecules, and are crucial for plant growth and development. Classically, phytohormones can be divided into five major classes, namely auxin, cytokinin, gibberellin, abscisic acid, and ethylene. In the past decades, new hormones such as brassinosteroids (Santner and Estelle, 2009) have been identified. I will focus here on two phytohormones to discuss in more details: auxin and ethylene.
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Many soil and plant-associated bacteria are capable to produce auxin. Of particular importance, the beneficial effect of plant growth promoting rhizobacteria (PGPR) is largely based on the production of auxin (Spaepen et al., 2007). A study on wheat showed that *Azospirillum brasilense* was able to shorten the root length and increase root hair formation because of its auxin production, and such morphology can be mimicked by addition of indole-3-acetic acid (IAA). However, mutant strains of *A. brasilense* could not induce the same morphological changes (Dobbelaere et al., 1999).

The ethylene is a phytohormone originally described as fruit-ripening and now broader roles in other processes have been suggested, such as opening of flowers (Ogawara et al., 2003), abscission of leaves (Beyer, 1975), and mediating root growth response to ambient temperature *via* the interference with auxin biosynthesis and transportation (Fei et al., 2017). But ethylene in high concentration can inhibit after germination and root elongation. The beneficial soil bacteria could reduce the level of ethylene *via* production of enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyzes ACC, the precursor of ethylene in plants (Hayat et al., 2010).

1.2.4.5 Interference with quorum sensing

The N-acyl homoserine lactone (AHL) signals are used by many bacteria to sense the density of local population and proximity of siblings, thereby bacteria can communicate between individuals which enables the coordination at group-level responses. A study in *Medicago truncatula* showed that AHL can alter the expression of a series of proteins, of which many are involved in defense and stress response (Mathesius et al., 2003). AHL-producing rhizosphere bacteria or AHL can induce systemic resistance in tomato (Schuhegger et al., 2006) and *Arabidopsis* (Schikora et al., 2011), respectively. Such reports implicate the role of AHL in the biocontrol of pathogens.

1.2.4.6 Volatile compounds

Volatile organic compounds (VOCs) represent an alternative way for microorganisms to interact with plants instead of direct contact. For instance, two VOCs, namely 2,3-butanediol and 3-hydroxy-2-butanone (acetoin), can be produced by some strains such as *Bacillus subtilis*, *Bacillus amyloquefaciens*, and *Enterobacter Cloacae*, and were shown to be beneficial for plant growth (Ryu et al., 2003). Later, other plant growth promoting VOCs such as 1-hexanol, indole, and pentadecane were identified (Blom et al., 2011). This
study also revealed that production of volatiles is widespread among rhizosphere bacteria but highly depends on culture conditions.

1.2.4.7 Biosynthesis of antimicrobial compounds

Rhizosphere represents the frontline defense for plant root against pathogen attacks. One of the mechanisms applied by rhizosphere microbiota to ward off pathogens is antibiotics (Raaijmakers and Mazzola, 2012). As for how penicillin was discovered (Foster and Raoult, 1974), microbes are able to produce antimicrobial compounds to act as “weapons” to survive in the ceaseless and numerous conflicts they engage (Davies, 1990). A series of such compounds have been identified such as 2,4-DAPG, pyoluteorin, pyrrolnitrin, cyclic lipopeptide surfactants, zwittermycin A, and bacteriocins (Emmert and Handelsman, 1999; Weller, 2007; Berg, 2009; Pérez-García et al., 2011). Of important, antimicrobial compounds exert antimicrobial activities in a concentration-dependent manner, acting as inhibitors at high concentration and as mediators of intercellular signaling at low concentrations (Davies et al., 2006; Fajardo and Martínez, 2008; Romero et al., 2011).

1.2.4.8 Induced systemic resistance

Induced systemic resistance (ISR) triggered by non-pathogenic rhizosphere microbes can protect the plant from a broad range of pathogens. ISR is a pre-treatment of plants with appropriate stimulus prior to pathogenic exposure enabling enhancement of defensive capacity (often called “priming effect”). The induction of ISR is mainly depending on two phytohormones which are jasmonic acid (JA) and ethylene (Choudhary et al., 2007), but salicylic acid (SA)-based pathway is also involved (van de Mortel et al., 2012). Some ISR-inducing microbes are well characterized including *Pseudomonas*, *Bacillus*, and *Serratia* species (De Vleesschauwer and Höfte, 2009).

1.2.5 Root microbiota in disease

Soil-borne plant pathogens such as nematodes, fungi, and bacteria can cause plant disease and reduce crop yield. According to a survey by the journal Molecular Plant Pathology, among the top 10 most scientific/economical bacteria, some of which infect plant via roots: *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, *Dickeya dadantii*, *Dickeya solani*, *Pectobacterium carotovorum* and *Pectobacterium atrosepticum* (Mansfield et al., 2012). However, the knowledge of the communication between plant host and pathogens is still
limited mainly due to the limits of analytical methods and complex conditions in the rhizosphere.

In addition to these pathogens, rhizosphere also harbors many potentially human opportunistic pathogens such as *Burkholderia cepacia* and *Stenotrophomonas maltophilia*, which are widely spread among different plants (Berg et al., 2005). Although the occurrence of pathogens in the rhizosphere is evidenced, very little information is available about the virulence relative to their clinical counterparts.

The presence of human pathogens within root tissue is also exemplified. For instance, *Escherichia coli* O157:H7, the cause of hemorrhagic diarrhea and Haemolytic uremic syndrome, has been isolated in a range of plants such as apples, sprouts, bean, cantaloupes, and leaf lettuce (Ackers et al., 1998; Hilborn et al., 1999). The transmission of *Escherichia coli* O157:H7 may be *via* the contaminated manure or irrigation water (Solomon et al., 2002).

### 1.3 Similarities between gut microbiota and rhizosphere microbiota

As discussed above, gut and rhizosphere are both open systems with a large exchange surface (microvilli for the gut and root hairs for the root) and overpopulated with tremendously diverse microbes (Fig. 4). Although gut and rhizosphere differ in microbial composition (Hacquard et al., 2015), these two systems are highly similar in microbial functions such as nutrient acquisition, protection against pathogens, modulation of immune system, microbial density and diversity.
INTRODUCTION

Figure 4. Comparisons between plant roots and human gut in terms of physiological functions, spatial distribution of microbiota, and factors driving community establishment. (A) and (B) refer to plant roots and human gut respectively. Upper panels are the principal nutrient fluxes and the chemical and biological gradients are depicted with bars with changing color or thickness. Lower panels are the compartments split horizontally along root axis or intestinal tract. Black bars refer to the range of compartments where different environmental or biological elements having an influence on microbial community establishment. Adapted from (Hacquard et al., 2015)

1.3.1 Nutrient uptake

Gut microbiota can assist break down of the glycan bonds and liberate additional energy from the diet during the fermentation which converts complex sugars, glycans to short-chain fatty acids (SCFAs). In addition, humans lack biosynthesis ability for most vitamins, but the gut microbiota stands as vitamin supplier (LeBlanc et al., 2013).
For plants, rhizosphere microbiota can solubilize phosphorus, uptake of iron via siderophores, weathering minerals, and degradation of recalcitrant organic matter (Berendsen et al., 2012; Bulgarelli et al., 2013). Arbuscular mycorrhiza increases the root surface significantly and plays important role in phosphorus uptake. For endophytes, rhizobia are well adapted to root tissue of legume plants and perform nitrogen fixation for the host.

1.3.2 Protection against pathogen

Gut microbiota protect human against pathogen infections via competing for nutrients, ecological niche, alteration of local pH, adhesion to receptors, production of inhibitory metabolites, and modulation of toxin production or action (Fagundes et al., 2012; Bulgarelli et al., 2013; Kamada et al., 2013). Similarly, rhizosphere microbiota can protect the host against pathogen via competition for nutrients, production of antimicrobial compounds and lytic enzymes (Berendsen et al., 2012; Doornbos et al., 2012).

1.3.3 Host immunity modulation

Gut microbiota are crucial in the induction, training, and function of host immune system. It is best exemplified by the germ-free mice that are functionally immature in many systems (Abrams et al., 1962; Smith et al., 2007). A list of bacteria have been shown to improve the symptoms in inflammatory bowel disease (IBD) in both human and mouse models (Round and Mazmanian, 2009). They are able to limit inflammation and disease mainly via regulatory T (T<sub>reg</sub>) cells.

Non-pathogenic rhizosphere microbiota can induce systematic resistance (ISR) to boost the defensive capacity of plants against a broad range of pathogens (Van der Ent et al., 2009). A myriad of bacterial traits, such as flagella, cell envelope components, can be recognized by plant innate immune system, thereby leading to a basal level of host defense (De Vleesschauwer and Höfte, 2009).

1.3.4 Microbiota density and diversity

The microbial density in the human gut is ranging from $1 \times 10^1$ bacterial cells per gram of content in the stomach to $1 \times 10^{12}$ in the colon which makes colon one of the most densely colonized microbial habitats (Whitman et al., 1998; Verdu et al., 2015). Gut is estimated to harbor over 1000 species which contains larger than 100 times of genes than the human
Rhizosphere, in a similar magnitude, is estimated to have $10^{11}$ cells per gram of root content (Egamberdieva et al., 2008) and populated by more than 30,000 prokaryote species (Mendes et al., 2011). Depending on the techniques, the number of operational taxonomical units (OTUs) range from less than 100 to more than 5500 based on a series of rhizosphere studies (Mendes et al., 2013).

### 1.3.5 Microbiota co-diversify with their host

As we have discussed above, gut and rhizosphere microbiota differ in phylogenetic composition, while they provide strikingly similar benefits to their host which could be due to the adaption to specific hosts and environmental features. In plants, host species or genotype has been shown to correlate with rhizosphere microbial communities (Rengel et al., 1996; Kuske et al., 2002; Micallef et al., 2009; Aira et al., 2010; Weinert et al., 2011; Peiffer et al., 2013; Bulgarelli et al., 2015). It suggests that the host’s evolutionary history can be a determinant of root microbiota structure (Bouffaud et al., 2014). Besides, the existence of core rhizosphere microbiota in *Arabidopsis* indicates that core ecological principles drive the assembly of root microbiota (Ramírez-Puebla et al., 2013). In addition to host-associated features, rhizosphere microbiota are also shaped and maintained by vertical transmission of endophytes *via* seeds (Puente et al., 2009; Hardoim et al., 2012; Barret et al., 2015; Johnston-Monje et al., 2016).

In mammals, gut microbial communities were shown to be influenced by mammalian phylogeny (Ley et al., 2008) and even to a stronger extent than diet (Amato et al., 2019). Irrespective of gender, age, and body mass index, a core microbiota (enterotypes) exist in the human gut (Turnbaugh et al., 2009; Arumugam et al., 2011; Burke et al., 2011). Furthermore, gut microbiota transmission from parent to offspring can also be acquired *via* the birth process, breast milk, and close contact that comes from parental care (Unger et al., 2015).

Collectively, gut and rhizosphere microbiota function as specialized symbionts and co-diversify with their host over time scales. Different host features and ecological filtering (environmental factors) between human and plants lead to different microbial composition, but in the perspective of coevolution (though it is controversial) (Moran and Sloan, 2015), they do function similarly.
2 Summary of manuscripts

Manuscript 1

We analyzed the short- (14 days) and long-term (13-39 months) effects of azithromycin administration on the gut microbiota of 59 children aged 12-36 months, diagnosed with recurrent asthma-like symptoms from the COPSAC2010 cohort. Each acute asthma-like episode was randomized to a 3-day course of azithromycin oral solution of 10 mg/kg per day or placebo. Fecal samples were collected and investigated by 16S rRNA gene amplicon sequencing. We found that:

- Short-term, azithromycin caused a 23% reduction in observed richness and 13% reduction in Shannon diversity. Microbiota composition was shifted primarily in the phylum Actinobacteria, especially a reduction of abundance in the genus Bifidobacterium.

- Long-term, we did not observe any differences in the gut microbiota composition between the azithromycin and placebo recipients.

We concluded that azithromycin treatment induced a perturbation in the gut microbiota 14 days after randomization but did not have long-lasting effects on the gut microbiota composition.

Manuscript 2

We performed a randomized, assessor-blinded, single-center clinical trial in a group of adults diagnosed type 2 diabetes. Patients were randomly allocated to either lifestyle intervention group (N=60, medication and intensive lifestyle intervention such as exercise, diet, and sleep etc.) or standard care group (N=26, medication and standard medical counseling) and followed up for 12 months. Fecal samples were collected before the intervention and every 3 months thereafter and assessed with 16S rRNA gene amplicon sequencing. We found that:

- The gut microbiota of patients in both groups changed massively over time.

- We observed no differences in the changes of alpha diversity, beta diversity, taxonomic composition, and functional composition between the two groups.

We concluded that the glucose-lowering medication drives the gut microbiota of diabetic patients in a similar way as the effects of lifestyle intervention. Besides, we speculate that
gut microbiota had limited influence on the improvements in physiological fitness for patients in the lifestyle intervention group.

**Manuscript 3**

We investigated the rhizosphere microbiota in multiple aspects including spatial distribution, the influence of individual variation and inoculation on microbial composition. We also compared the influence of sampling scale: one in the scale of root sections along root axis; one in the scale of an entire root system. We found that:

- Root tip is lower in richness, diversity, and evenness compared to middle section and root base.
- Root tip is enriched with Firmicutes, while Patescibacteria and Verrucomicrobia were more abundant in root base.
- Plant identity (between replicates) was a strong influencing factor on rhizosphere microbiota.
- The influence of a microbial inoculum applied to the seedlings on rhizosphere microbiota was mainly maintained at root base instead of root tip and middle section.
- Sampling at larger scale (entire root system) yielded more stable sample types in terms of alpha and beta diversity compared to fine-scale sampling (in the scale of root sections).

In conclusion, rhizosphere bacteria were highly heterogeneous along the root axis, with more stable communities in older and superficial parts while deeper and younger parts were more variable. Despite genetic homogeneity, plant individual variation played a significant role in shaping the root microbiota. The inoculated root microbial communities were better maintained at root base than root tip. Sampling at the scale of an entire root system was naturally limited in detecting the microbial inoculation effects.
3 Discussion

This thesis investigated two extremely complex ecosystems, namely the human gut and plant rhizosphere microbiota. We addressed the influence of factors on their associated microbiota, such as host physical fitness, medication use, sampling scales, individual variability, and inoculation. I will discuss the results of three manuscripts from the perspective of medication and sampling scales.

3.1 Medication induced alteration in microbial composition

The side-effects of medications are a frequently raised concern, many of which are related to the digestive system. Therefore, using high throughput sequencing techniques has been a hot topic for such investigations.

Manuscripts 1 and 2 in this thesis assessed the influence of medication on the gut microbiota in two different clinical situations: 1) Young children prescribed azithromycin for recurrent asthma-like episodes; 2) Adults prescribed glucose-lowering medications (mainly metformin) to lower blood glucose level.

Azithromycin was shown to have a strong short-term influence on the gut microbiota, but such an effect did not last long in young children. Our study is strengthened by the RCT study design, in addition to which, both short- and long-term effects of azithromycin were addressed. Our results showed that *Bifidobacterium* was the most vulnerable genus to azithromycin and its abundance was reduced massively short after treatment. *Bifidobacterium* is well known to have health benefits for the host and has long been used as probiotics to prevent or treat diseases (Sanders et al., 2010). For example, *Bifidobacterium* has been shown to reduce the lipopolysaccharide (LPS) level and improve gut barrier function (Griffiths et al., 2004; Wang et al., 2006). Therefore, a tremendous reduction of this genus in a short time may potentiate health problems. Fortunately, we found that azithromycin did not induce long-lasting reduction of *Bifidobacterium* and its abundance recovered to the same level as the control group.

A limitation in our study was that we did not screen potential antibiotic resistance genes as a potential long-term effect; however, we recognize that this issue should be addressed.

Glucose-lowering medication and healthy lifestyles are two basic principles to manage type 2 diabetes (T2D) (World Health Organization, 2016), but their comparative influence on gut microbiota is unclear. In our study, we found that glucose-lowering medication,
especially metformin, increased gut microbial diversity significantly. In literature, conflicting results have been reported about the influence of metformin on gut microbial diversity. Tong et al. (2018) found that 12 weeks of metformin treatment in humans increased Simpson diversity. From a group of 784 human gut metagenomes, Forslund et al. (2015) reported an increase of gut microbial richness due to metformin treatment, but this was not significant. Opposite results have also been observed where metformin treatment decreased Shannon diversity (Sun et al., 2018). Such divergences might be derived from the study population, where Tong and Forslund’s studies were diabetic subjects, but Sun’s study population was metformin naive and newly diagnosed T2D.

Lifestyle intervention (exercise and diet) is another management for T2D patients. Several studies have shown that physical exercise can increase gut microbial diversity both in human and animals (Chen et al., 2018). For instance, a study showed that athletes (exercise and diet) had a higher diversity of gut microbiota than healthy controls (Clarke et al., 2014), this greater diversity is associated with better health status and negatively correlates with metabolic risk marker (Shanahan, 2010; Brahe et al., 2015). To our knowledge, we are the first study to compare the effects of glucose-lowering medication vs. lifestyle intervention on the gut microbiota in a longitudinal design. We observed parallel development of gut microbiota in terms of alpha diversity, beta diversity, taxonomic composition, and functional composition in the patients from both groups regardless of their different improvement in physical fitness. Therefore based on our data, we conclude that glucose-lowering medication drives the gut microbiota in a similar direction as the effect of lifestyle intervention and speculate that gut microbiota are not deeply involved in the improvement of physical fitness.

3.2 Sampling scale in rhizosphere studies

Traditionally, a rhizosphere sample is the mixture of an entire root system from a plant (Barillot et al., 2013). Through this method, the rhizosphere effect has been well-observed. The plant roots are known to be heterogeneous in terms of host metabolism, root structure, physical, chemical and biological characteristics (Hinsinger et al., 2009; Carminati, 2013; Razavi et al., 2016; Kreuzeder et al., 2018). Therefore, to accurately decipher these variations along root axis, a smaller sampling scale is needed. In manuscript 3, we compared different sampling scales in elucidating rhizosphere microbiota by either sampling at the scale of an entire root system, or sampling at the scale of a single root. At the small scale, high heterogeneity of rhizosphere microbiota was observed, such as alpha
diversity, beta diversity, and microbial distribution. Importantly, we also observed the heterogeneous colonization of inoculants along root axis where root base was the hotspot for strong interaction between plant roots and inoculum and a few newcomers from inoculant successfully colonized this root section. But naturally, these fine scale variations were not detected through traditional large sampling scale. Our results highlighted the significance of fine scale sampling since the traditional sampling methods are often hard to detect the inoculation effect likely due to inappropriate sampling strategy.
4 Perspectives

In manuscript 1, we addressed the short- and long-term effect of azithromycin on gut microbiota in young children. We found that azithromycin had a strong influence on gut microbiota for short-term, but a long-lasting disturbance was not observed. However, our results lack the investigation of antibiotic resistance genes. Most bacteria are resistant to antibiotics (D’costa et al., 2006), it is a feature of bacteria evolved by millions of years of direct or indirect exposure to antibiotics (D’Costa et al., 2011). But the intensive use of antibiotics in model society functions as a selection of pre-existing antibiotic resistance genes. Korpela et al. (2016) showed resistance genes can persist 6-12 months after treatment and even a longer time was observed in other studies (Table 1). Microbes may represent undisturbed abundance long after antibiotics treatment, but potentially become enriched with antibiotic resistance genes that lead to the reduced drug efficacy (Ventola, 2015). Therefore, future work addressing the disturbance of azithromycin at the gene level will substantially complete the missing knowledge.

In manuscript 2, we compared the influence of two therapies on the gut microbiota for T2D patients: one was glucose-lowering medication based, the other was lifestyle intervention based. Because the lifestyle intervention included a few lifestyle elements, we are not able to interpret the individual effect. Besides, we are not able to clearly differentiate the effect of medication and lifestyle. Our results so far are observations, future work is to find out the true determinants that leading to a similar gut microbial development between groups.

In manuscript 3, we investigated the rhizosphere microbial communities in different scales and highlighted the necessity of fine scale sampling to observe the inoculation effect. Our results showed the uneven distribution of rhizosphere microbiota and how they respond differently to inoculation, indicating heterogeneity of microbial interactions along the root axis. Therefore, it will be interesting to investigate the correlation-based network to compare the mutualistic and antagonistic relationships at different root sections. Furthermore, we can assess the strength of priority effects by predicting the microbial assembly in one root section based on the microbial composition in another root section.
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Appendix

Manuscript 1
Research paper

Short- and long-term impacts of azithromycin treatment on the gut microbiota in children: A double-blind, randomized, placebo-controlled trial

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A B S T R A C T

Background: Macrolides are commonly prescribed for respiratory infections and asthma-like episodes in children. While their clinical benefits have been proved, concerns regarding the side-effects of their therapeutic use have been raised. Here we assess the short- and long-term impacts of azithromycin on the gut microbiota of young children.

Methods: We performed a randomized, double-blind, placebo-controlled trial in a group of children aged 12–36 months, diagnosed with recurrent asthma-like symptoms from the COPSAC2010 cohort. Each acute asthma-like episode was randomized to a 3-day course of azithromycin oral solution of 10 mg/kg per day or placebo. Azithromycin reduced episode duration by half, which was the primary end-point and reported previously. The assessment of gut microbiota after treatment was the secondary end-point and reported in this study. Fecal samples were collected 14 days after randomization (N = 59, short-term) and again at age 4 years (N = 49, long-term, of whom N = 18 were placebo treated) and investigated by 16S RNA gene amplicon sequencing.

Findings: Short-term, azithromycin caused a 23% reduction in observed richness and 13% reduction in Shannon diversity. Microbiota composition was shifted primarily in the Actinobacteria phylum, especially a reduction of abundance in the genus Bifidobacterium. Long-term (13–39 months after treatment), we did not observe any differences between the azithromycin and placebo recipients in their gut microbiota composition.

Interpretation: Azithromycin treatment induced a perturbation in the gut microbiota 14 days after randomization but did not have long-lasting effects on the gut microbiota composition. However, it should be noted that our analyses included a limited number of fecal samples for the placebo treated group at age 4 years.

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1. Introduction

There has been a rapid rise in the use of antibiotics over the past decades [1]. Respiratory infections account for the majority of hospital visits during which antibiotics are prescribed [2]. Even though current guidelines do not recommend antibiotics for the treatment of asthma-like episodes in young children [3], they are among the most commonly prescribed drugs for this condition [4]. Macrolides are often prescribed to children in USA [5], especially to those with respiratory infections and penicillin allergies [6–9]. They are considered safe, well-tolerated and possess antimicrobial activity against gram-positive cocci, such as Streptococcus pneumoniae, and gram-negative cocci Moraxella catarrhalis and atypical pathogens such as Mycoplasma pneumoniae [7]. Besides these activities, azithromycin, a second-generation macrolide, shows antimicrobial activity against microorganisms that erythromycin has no or marginal effect on such as Haemophilus influenzae [10]. We recently reported a reduction in the duration of asthma-like symptoms by half after azithromycin treatment [11]. However, the use of antibiotics for reducing such episodes in children does raise concerns given the worldwide action plans to reduce per capita antibiotic consumption. On the one hand, it has been well documented that antibiotic consumption is the primary driver of antibiotic resistance...
Research in context

Evidence before this study

Findings from our previous studies showed that antibiotics such as azithromycin could shorten the duration of asthma-like symptoms in young children. While the clinical benefits of azithromycin intervention have been proved, the potential drawbacks of its use still remain. Considering the associations of gut microbiota with health problems, it is important to investigate the potential consequences introduced to the gut microbiota when azithromycin is prescribed in clinic. On Feb 4, 2018, we searched the scientific literature in PubMed (with no date or language restrictions) for the various combinations of the following search terms "antibiotics", "RCT", "intestinal", and "gut". We identified all previous studies regarding the influence of antibiotics on the gut microbiota in children. Only few publications were double-blind, randomized, placebo-controlled trial (DB-RCT) design, among which none had investigated the long-term effect of antibiotic administration on gut microbiota.

Added value of this study

This study, to our knowledge, is the first DB-RCT investigating both short- and long-term impacts of azithromycin treatment on the gut microbiota composition in children. These data showed a massive perturbation of gut microbiota composition shortly after azithromycin treatment, but the long-lasting adverse effects regarding such perturbations were not observed. However, our analyses did have a limited number of fecal samples for the placebo treated group at age 4 years.

Implications of all the available evidence

Even though our previous study proved the clinical benefits of azithromycin treatment, current guidelines do not recommend antibiotics for the treatment of asthma-like episodes in young children. Compared to the clearly observed disturbance of the gut microbiota composition shortly after azithromycin treatment, its long-term effects regarding such disturbance were not detected. These findings suggested that antibiotic intervention is a strong factor in influencing the gut microbiota for short-term, but the general concerns regarding the undesired, long-lasting impact are alleviated. However, for long-term effects, we were able to analyze only a limited number of fecal samples for the placebo treated group (N = 18) at age 4 years. Nevertheless, the impact of azithromycin treatment at the gene level, such as the gut resistome, and the correlations of such treatment with health problems later in life need to be investigated.

[12,13], and can lead to dysbiosis of the gut microbiota [14]. On the other hand, the treatment of recurrent asthma-like episodes in children represents a major unmet clinical need that has an impact on both the children’s quality of life and healthcare resources. Naturally, the benefits and potential drawbacks of antibiotic use for acute management of asthma-like episodes represent a clinical dilemma. Whilst azithromycin is efficient at reducing episodes duration in young children with recurrent asthma-like symptoms, its potential long-term impact on the development of gut microbiota needs to be addressed.

The gut microbiota of adults is a complex and relatively stable community, involved in both host metabolic activity [15] and immune function [16]. However, the taxonomic composition and the structure of this community is highly variable during the first 2-3 years of life [17] and is continuously influenced by numerous factors [18–20], of which antibiotic use is suggested to have the most profound effects [21]. Trasande et al. [22] found that the earlier in life an antibiotic is prescribed, the greater its influence on body mass index (BMI). Studies have previously shown that the gut microbiota is important during the first year of life, as reduced diversity was associated with increased risk of allergic disease [23–25] and delayed maturation can trigger an inherited asthma risk [26]. Alterations of the gut microbiota during this critical window have been suspected to have long-lasting consequences [27], such as decreased richness of the gut microbiota [28]. Although the bacterial richness can recover rapidly in adults [29], high level of antibiotic resistance genes are still observed years later [30,31]. Furthermore, antibiotics can potentially induce the enrichment of antibiotic resistant strains [32], pathogen invasion facilitated by perturbation of non-target commensal gut microbes [33], and community-wide alterations in the gut microbiota composition [34].

Recently, two double-blind, randomized, placebo-controlled trials (DB-RCTs) have investigated the short-term impact of azithromycin treatment on the gut microbiota in children. Both studies found a decrease in richness and diversity of the gut microbiota and an altered taxonomic composition [35,36]. In contrast to the short-term impact of azithromycin on the gut microbiota in children, its long-term effects are not well known. One observational study suggested influences on children’s gut microbiota for up to 2 years after macrolide treatment (s) [34]. We therefore explored these effects in a nested DB-RCT in the unselected Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC2010) mother-child cohort [37]. Here, we investigate both short-term and long-term impact of azithromycin treatment on the gut microbiota in children. Our study aims to clarify concerns regarding the disturbance of gut microbiota composition when using azithromycin for acute management of recurrent asthma-like episodes in young children.

2. Materials and methods

2.1. Study design and participants

As part of the COPSAC2010 cohort, the parents filled out a structured symptoms diary of their children’s airway symptoms every day from birth. Parents of children, aged 12–36 months, were invited to participate in the DB-RCT if diagnosed with recurrent asthma-like symptoms, defined as: five episodes of troublesome lung symptoms within 6 months; 4 weeks of continuous symptoms; a severe acute episode needing oral prednisolone or hospital admission. Exclusion criteria were macrolide allergy, heart, liver, neurological, kidney disease and or one or more clinical signs of pneumonia. More details regarding cohort enrollment can be found in our previous publication [11].

Children participating in the DB-RCT were prescribed a 3-day course of oral azithromycin solution of 10 mg/kg per day or matching placebo at acute asthma-like episodes from 12 to 36 months and fecal samples were collected 14 days after randomization and no baseline samples were collected before treatment (Fig. 1a). Children could be included in the trial at a maximum of seven asthma-like episodes, with each treatment randomized independently of any prior treatments. Because participations of children in the DB-RCT were episode driven, children would be invited to participate again if they experienced later episodes. Therefore, additional participations in the trial occurred after a random time interval. Fecal sample was collected from each child when they were 4 years old in the same manner [26].

2.2. Study population

In the DB-RCTs, a total of 72 children (mean age 2-0 years [SD 0-6]) were recruited, each with one to seven episodes (Fig. 1). A total of 124 fecal samples from 62 children were received. After removing eight samples due to low sample quality, the remaining 116 samples from
59 children were considered as the sample population in the DB-RCTs. Since some children participated in the DB-RCT more than once, to avoid within-child correlation, only samples collected at the first participation were used for short-term analysis and were grouped based on how children were treated (azithromycin or placebo) (Fig. 1b). Among these 59 children, at their first participation, 29 were from the azithromycin-treated (AZT) group and 30 were from the placebo group. The baseline characteristics of recruited children in the DB-RCT are shown in Supplementary Table 1 and none of the clinical covariates differed significantly between treatment groups.

At 4 years of age, fecal samples were collected from 49 children; of which 31 samples were from children who were treated with azithromycin at least once during the DB-RCTs and the remaining 18 samples were from children who only received placebo (Fig. 1b).

2.3. Randomization and masking

Each asthma-like episode was randomized to either azithromycin or placebo. Treatments were randomly allocated at the Pharmacy of Glostrup (Copenhagen, Denmark) with the computer generated random numbers in blocks of ten. The copies of randomized code were kept at the research site and the pharmacy in sealed envelopes. Investigators and participating families were masked to treatment assignment until children turned 3 years old. Those assessing primary outcome were masked; those doing secondary outcome, which is presented in this study, were not.

2.4. DNA extraction, sequencing, and bioinformatic analysis

DNA extraction and sequencing were performed as described by Mortensen et al. [38] Briefly, the microbial DNA was extracted using the PowerMag® Soil DNA Isolation Kit on the EpMotion® automated pipetting system. EpMotion 5075 (Eppendorf). The microbiota was investigated by 16S rRNA gene sequencing using a two-step PCR procedure targeting the V4 region (~290 bp; primers 515F [5'-GTCCACGCMGCCGCGTAA-3'] and 806R [5'-GGACTACHVGGGTWTCTAAT-3']). Paired-end sequencing (2 × 250 bp) was performed on the Illumina MiSeq System (Illumina Inc., CA, USA) with the MiSeq Reagent Kits v2 (Illumina Inc., CA, USA); 5-0% PhiX was included as an internal control.

Bioinformatic analysis was performed as described by Stokholm et al. [26] Briefly, the raw Illumina MiSeq sequencing output was primer trimmed (biopieces), quality filtered and merged (UPARSE), and de-novo operational taxonomic unit (OTU) clustered at 97% (vsearch). A phylogenetic tree was built (QILME) and the taxonomy was predicted against the Greengenes database (version of 2013).

We used rarefaction curves to determine the minimum sequencing depth necessary to describe the microbiota of each sample (Supplementary Fig. 1). The rarefaction curves showed that Shannon diversity reaches asymptote for samples at 1000 sequences. Based on this, samples with less than 2000 sequences were excluded.

2.5. Statistical analysis

Continuous and categorical data of baseline characteristics were analyzed with t-test and chi-square test respectively. The sample size of this study was estimated based on the primary end-point (episode duration) and has been reported previously [11].

The effect of azithromycin on alpha diversity (Shannon index and observed richness) was assessed with two linear regression models (function “lm” in R-package “stats”): one for short-term effect of azithromycin treatment (14 days after randomization, at the first participation), age of a child was included as a covariate; one for long-term
effect (4 years of age), number of times a child participated in the DB-RCT was included as a covariate. To fulfill the assumptions of linear regression, Shannon index at 4 years of age was transformed with “boxcox” in R-package “MASS” because of the violation of normality. For beta diversity, comparisons of UniFrac distances (R-package “phyloseq”) between groups were tested with Permutational Multivariate Analysis of Variance with adonis (R-package “vegan”) (treatment and age were included as variables) [39,40]. Comparisons of relative abundance of taxa at all phylogenetic levels between treatment groups were assessed with permutation test [41].

To identify genera that were most correlated with treatment, a Random Forest model (named as “RF-1”) was performed at genus level (R-package “randomForest”) [42]. Its performance was validated via 20 cycles of 10-fold cross-validation (200 iterations in total), with 5000 trees per iteration. The parameter “mtry” was tuned by 10 cycles of 10-fold cross-validation (100 iterations in total) of all possible values.

To assess the recovery of gut microbiota, we built two Random Forest models at OTU level based on fecal samples collected at the first participation (RF-2 model) and 4 years of age (RF-3 model). These two models were performed with 5000 trees and the default value of parameter “mtry”. The prediction accuracy of Random Forest models was obtained from the confusion matrix and Area Under the ROC Curve (AUC) was calculated.

2.6. Governance

The COPSAC2010 study was approved by the Local Ethics Committee for Copenhagen (H-8-2008-093) and the Danish Data Protection Agency (2015–41–3696). This DB-RCT was approved separately by: the Local Ethics Committee (H-3-2010-065), the Danish Data Protection Agency (2010–41–5023); the Danish Health and Medicines Authority (2612–4329), and registered at ClinicalTrials.gov (NCT01233297). Parents of children gave written and oral informed consent before enrolment of participants. The complete COPSAC biobank is publicly available at the Danish National Biobank (www.biobankdenmark.dk). The entire COPSAC data, including the DB-RCT specific data, are currently being transferred to a publicly available database (the Danish Data Archive, www.sa.dk).

3. Results

3.1. Short-term: alteration of alpha and beta diversity at day 14

At day 14, after randomization, 30 AZT children had significantly lower richness in the fecal samples compared to the 29 placebo children (177.8 ± 56.0 [mean ± standard deviation] vs. 230.6 ± 61.2, respectively, p = 0.0006; Fig. 2). Similarly, Shannon diversity was significantly lower in the AZT group compared to the placebo group (2.96 ± 0.80 [mean ± standard deviation] vs. 3.41 ± 0.58, respectively, p = 0.009). Both alpha diversity indices increased over age, during which the discrepancies in diversity between groups reduced.

Based on UniFrac distance, the principal coordinates analysis (PCoA) plot illustrated that the AZT group partially overlapped with the placebo group; treatment accounted for a small but significant proportion of variance (R² = 3.8%, p = 0.027 and R² = 4.2%, p = 0.0007, weighted and un-weighted distance, respectively; Supplementary Fig. 2).

3.2. Short-term: alteration of taxonomic composition at day 14

Bacteroidetes and Firmicutes were the most abundant phyla (relative abundance 57·7% and 31·6%, respectively), followed by Proteobacteria, Actinobacteria, and Bacteroidia; these five phyla had a combined

![Fig. 2. Short-term effect: Alpha diversity over age between groups. Distribution of observed richness and Shannon diversity for the AZT (red lines) and the placebo (blue lines) groups. The line indicates the linear regression of the correlation between age and alpha diversity.](image-url)
relative abundance of 99.7%. We observed a decrease in the relative abundance of Actinobacteria in the AZT group compared to the placebo group (Supplementary Table 2). Notably, Bifidobacterium accounted for the majority of composition changes in Actinobacteria, which was evident at all taxonomic ranks, particularly OTU level, where 17 of 21 significant OTUs belonged to Bifidobacterium and all were dramatically reduced in the AZT group.

3.3. Short-term: random forest models based on taxonomic composition at day 14

To further elucidate the impact of azithromycin treatment on the gut microbiota composition and to identify its recovery purely based on the gut microbiota, we built two Random Forest models, a supervised machine-learning algorithm, based on the 59 samples collected at the first participation. The first model (RF-1), built at genus level, produced an AUC of 0.89 (p = 0, by permutation test with 10,000 iterations), and was used to identify genera that were most affected by azithromycin treatment. The genera having best treatment-discriminatory performance were identified based on importance scores (Fig. 3), among which Bifidobacterium showed an exceedingly higher score than the remaining genera. The second model (RF-2), built at OTU level, produced an AUC of 0.92 (p = 0, by permutation test with 10,000 iterations), and was used to assess the recovery of gut microbiota at the second participation and 4 years of age.

3.4. Second participation: partial recovery of gut microbiota

After the first randomization, 28 children fulfilled the inclusion criteria again and participated in the DB-RCT for their second time and also had a fecal sample collected. Although the time intervals between two participations were variable (mean 223.3 days [SD 152.8]), the relatively longer time than 14 days enabled us to assess the recovery of gut microbiota after azithromycin treatment; 11 of these 28 children were treated with placebo at their second randomization, and the effect of their first treatment could be evaluated here. Among these 11 children, six were treated with azithromycin, and five were treated with placebo at their first randomization. No difference between groups was observed in either alpha diversity (median of observed richness, 183 ± 74.5 vs. 233 ± 64 for AZT and placebo, respectively, p = 0.52, Wilcoxon rank-sum test; median of Shannon, 3.43 ± 0.89 vs. 3.94 ± 0.47 for AZT and placebo, respectively, p = 0.13, Wilcoxon rank-sum test) or beta diversity (weighted Unifrac, R² = 12.8%, p = 0.22). Next, we applied RF-2 model to assess the recovery of gut microbiota and we correctly identified the treatments for three of six samples in azithromycin-treated group and five of five samples in placebo group (AUC = 0.94, p = 0, by permutation test with 10,000 iterations). The prediction with Random Forest model indicated that half of the children who were treated with azithromycin at their first participation did not recover within this time interval.

![Fig. 3. Short-term effect: The top 20 taxa with the highest importance score (Gini index) by the Random Forest algorithm for distinguishing treatment groups and their corresponding relative abundances.](image-url)
3.5. Long-term: recovery of gut microbiota at 4 years of age

To assess the long-term impact of azithromycin, we investigated the 49 fecal samples collected from AZT (N = 31) and placebo (N = 18) groups when children were 4 years old. We did not observe any significant differences in alpha diversity (mean of observed richness, 181.5 ± 49.9 vs. 188.9 ± 41.2 for AZT and placebo, respectively, p = 0.66; mean of Shannon, 3.47 ± 0.72 vs. 3.64 ± 0.44 for AZT and placebo, respectively, p = 0.90; Fig. 4a) or beta diversity between groups (weighted UniFrac, R² = 2.0%; p = 0.37; Fig. 4b). Furthermore, we did not observe any OTUs differ significantly in relative abundance between groups. Next, the RF-2 model was applied to assess the recovery of these children based on the gut microbiota. Of the 31 children in AZT group, 26 were identified as placebo, resulting in an AUC of 0.69 (p = 0.013, by permutation test with 10,000 iterations). To further validate the result, we built a third Random Forest model for 4-year samples at the OTU level (RF-3 model) to differentiate the treatment groups. The RF-3 model produced an AUC of 0.56 (p = 0.24, by permutation test with 10,000 iterations).

4. Discussion

Azithromycin had a strong effect on the composition of gut microbiota 14 days post-treatment, but these effects did not persist to 4 years of age (13–39 months after the last treatment) in our DB-RCT of azithromycin in young children [11]. Current guidelines discourage the use of antibiotics during asthma-like episodes in early life due to lack of evidence of severe bacterial infections as main episode triggers [3], and adverse effects on the colonizing microbiota [43,44]. Recent evidence showed that bacteria are important triggers for asthmatic episodes [45], and that azithromycin reduced the duration of symptoms by half [11].

In the present study, the 3-day course of azithromycin resulted in a perturbation of the gut microbiota 14 days after randomization. Alpha diversity was significantly reduced and the microbiota composition was shifted. However, long-lasting impact of azithromycin on the gut microbiota composition was not observed.

In our study, 14 days after randomization, children in the AZT group had 23% lower richness and 13% lower Shannon diversity in their fecal samples compared to the placebo group. In particular, the relative abundance of *Actinobacteria* was reduced. Based on the taxonomic composition, the Random Forest model identified study arms with high accuracy, the genus *Bifidobacterium* was the most important contributor.

We observed increasing richness and Shannon diversity with age of the child, which represented an ongoing maturation of the gut microbiota. Of interest, the later the azithromycin prescribed to children, the smaller the difference in alpha diversity seemed between two treatment groups. This decreasing discrepancy may be attributed to early antibiotic administration having stronger microbiota perturbing effects in younger children where the microbiota is still developing [22] compared to the older children, who may recover faster because of a more mature baseline composition. However, this study did not provide sufficient statistical power to confirm a significant interaction between age and treatment, therefore further investigation is needed.

Long-term effects of azithromycin treatment were not observed. At 4 years of age (13–39 months after the last treatment), we could not distinguish children according to AZT or placebo group based on alpha
diversity, beta diversity, discriminant OTUs or by Random Forest models. The full recovery of children’s gut microbiota in AZT group indicated that azithromycin treatment did not induce long-term compositional perturbations.

Our results are at odds with an observational study of children on the influence of macrolides on gut microbiota [34]. They observed lower richness for subjects who were exposed to macrolides within the preceding 2 years compared to the control group. The discrepancy may derive from some differences existing between our data and that of Korpela et al. The recovery time (13–39 months) of our subjects is longer compared to theirs (12–24 months); the age (median 2.0 years [IQR 1–0]) of our subjects is younger compared to theirs (median 5 years). Furthermore, observational studies may always have additional confounding factors, which drive both antibiotic use and microbial differences.

Bifidobacterium, the dominant genus in Actinobacteria, was one of the most affected genera by azithromycin treatment and had an exceedingly high importance score determined by Random Forest model. The relative abundance of Bifidobacterium in the AZT group was 50-fold lower compared to the placebo group and in many cases they were too low to be detected. Bifidobacterium has been shown to be one of the most affected genera by clarithromycin and metronidazole in the gut [31]. Most of the Bifidobacterium spp. strains are likely susceptible to macrolides and other antibiotics [46]. Similar results were observed in Korpela’s study where the abundance of Bifidobacterium was reduced around 4-fold when a participant was treated with macrolides during the preceding 6 months. However, two recent DB-RCTs found no difference in Bifidobacterium abundance between groups [35,36]. These discrepancies may derive from the different characteristics of study population, since Parker’s and Doan’s populations were from South India and Niger, respectively, compared to our cohort from Denmark.

Our results revealed that azithromycin treatment for asthma-like symptoms in childhood led to a transient perturbation of the gut microbiota composition (N = 59, 12–36 months of age); however, long-term impact of azithromycin regarding such perturbations was not observed (N = 49, 4 years of age). Our study may alleviate concerns about adverse effects of azithromycin use in young children. Furthermore, considering the strength of DB-RCT and azithromycin likely being the main source of disturbance on gut microbiota, we speculate that our findings may also extend to non-asthmatic children (12 to 36 months of age) who have been prescribed azithromycin.

However, limitations should also be acknowledged. The children may have received antibiotics for other reasons during these first 4 years of life, but that would work against the null hypothesis. Even though we had 116 samples from the 12–36 months period, in order to avoid within-child correlations, only 59 samples from the first randomization were used for short-term analysis. Therefore, we may have low statistical power to distinguish the differences between treatment groups. A similar issue for the 4-year samples was that most children had been randomized to azithromycin at one point during the trial period, reducing the size of the placebo group compared to the AZT group. In addition, exclusions and loss to follow up also resulted in the reduction of sample size. Furthermore, since we did not collect baseline samples before randomization, we could only assess the alteration of gut microbiota at the group level instead of tracking individual child before and after treatment. For the recovery assessment of the gut microbiota at the second participation, we were limited by both a small sample size and variable time intervals between participations. Another limitation was the resolution of 16S rRNA gene sequencing techniques and perturbation caused by azithromycin at the gene level, such as antimicrobial resistance, could not be evaluated. Most OTUs were classified to genus level, but for some OTUs the resolution was insufficient for such classification, therefore the unclassified taxa might introduce bias for statistical analysis.

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Declaration of interests

HB reports personal fees from Chiesi Pharmaceuticals, outside the submitted work. All other authors have nothing to disclose.

Author contributions

HB designed and carried out the study. SJS supervised the data acquisition. SW, MSM, ADB, JT, and MAR contributed to the statistical analysis. SW, MSM, JSs contributed to the concept and interpretation of the data. SW drafted the manuscript, UT contributed to the writing and preparation. All authors made a substantial contribution in the revision of the manuscript.

Availability of data and material

The datasets analyzed and/or used in the present study are available from the corresponding author upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.11.035.

References

Manuscript 2
Glucose-lowering medication and lifestyle intervention drive the gut microbiota in a similar direction: A randomized clinical trial

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Abstract
General type 2 diabetes management is based on two approaches, glucose-lowering medication and healthy lifestyles. The medication use raises concerns regarding their potential adverse effects, such as dysbiosis of the gut microbiota. Here we assessed the impact of glucose-lowering medication (first-line biguanide [tablet Metformin] and second-line GLP-1-analogue liraglutide [Victoza]) on gut microbiota compared to the effect of lifestyle intervention for diabetic patients. We performed a randomized, assessor-blinded, single-center clinical trial in a group of adults diagnosed with type 2 diabetes. All patients received standard care including medical counseling, education in type 2 diabetes, and lifestyle advice. They were randomly allocated to either standard care group (N=26) or lifestyle intervention group (N=60) where patients additionally received intensive physical exercise and dietary plans aiming to lower their body mass index. All patients were followed for 12 months. Fecal samples were collected at the study initiation, every 3 months thereafter and assessed with 16S rRNA gene amplicon sequencing. The gut microbiota of patients in both groups changed massively over time. Nevertheless, neither alpha diversity, beta diversity, taxonomic composition, nor functional composition developed differently between groups. We speculate that glucose-lowering medication drives the gut microbiota of diabetic patients in a way similar as the effect of lifestyle intervention. It indicated that gut microbiota had limited influence on the improvements of physiological fitness for patients in the lifestyle intervention group.
**Introduction**

Type 2 diabetes (T2D) prevalence has risen rapidly over the past decade(s) and become a global health issue (Dabelea et al., 2014; World Health Organization, 2016). Glucose-lowering medication, such as the first-line medication metformin and Glucagon-like peptide 1 (GLP-1) analogues, are effective at lowering blood glucose but how they impact the gut microbiota is unclear (Inzucchi et al., 2012; Gupta, 2013). Delayed-release metformin was shown to be more effective at glycemic control compared to immediate-release or extended-release metformin, indicating that metformin activity occurs in the gut (Buse et al., 2016). The concentration of metformin is 300 times higher in the gut than that in plasma (Bailey et al., 2008) and a more pronounced glucose lowering effect was observed when administered orally than intravenously (Stepensky et al., 2002).

Although the benefits of glucose-lowering medications have been proved in clinic, concerns regarding their adverse effects, such as discomforts and decreased quality of life, have been raised (Hoffmann et al., 2003; Huang et al., 2007; Prasad-Reddy and Isaacs, 2015). Compositional and functional shifts in gut microbiota have been reported in T2D patients following metformin treatment, such as increased relative abundance of *Escherichia* spp. and *Akkermansia muciniphila*, and depletion of *Intestinibacter* spp., and enhanced butyrate and propionate production potential (Shin et al., 2014; Forslund et al., 2015). Recently, strong effects of metformin on the gut microbiota were identified in a group of treatment-naive T2D individuals and the relative abundance of 81 and 86 bacterial strains were shifted (Wu et al., 2017). Sun *et al.* (2018) found that metformin decreased the alpha diversity of gut microbiota and substantially reshaped microbial community structures. The GLP-1 receptor agonist liraglutide (Victoza) has been shown to shift gut microbiota in mice by increasing the lean-related gut microbiota (phylum Firmicutes) and decreasing obesity-related microbiota (phylum Bacteroidetes) (Zhao et al., 2018). Besides, liraglutide treatment was linked to the increase of *Akkermansia muciniphila* and reduction of Proteobacteria (Moreira et al., 2018).

Lifestyle intervention, characterized by increased physical activities and dietary changes etc., is often suggested for diabetic patients, especially for those who are obese and/or insulin resistant (Care, 2013). The physiological benefits of such intervention include enhanced fuel mobilization, muscle glucose uptake, and fat oxidation (Li et al., 2004). A number of studies have reported lifestyle intervention can reduce the incidences of T2D (Knowler et al., 2002; Ramachandran et al., 2006; Gong et al., 2011), prevent further
deterioration of impaired glucose tolerance (Tuomilehto et al., 2001; Ramachandran et al., 2006), decrease the HbA1c levels (Umpierre et al., 2016), reduce the medication use (Johansen et al., 2017), and is regarded as effective as the medication treatment (Gillies et al., 2007).

We previously reported the primary outcomes of the 12-month lifestyle intervention, we found increased physiological fitness and reduced medication use for subjects in the intervention group (Johansen et al., 2017). In this study, we analyzed the gut microbiota changes (secondary outcome) and investigated the influence of two treatment therapies (lifestyle intervention vs. standard care).

**Methods and materials**

**Study design and participants**

To assess the effect of lifestyle intervention on glycemic control, a total of 86 patients, mean age of 54.3 years [SD, 9.0], were recruited via media and Danish Diabetes Association. Inclusion criteria were: above 18 years old, type 2 diabetes for less than 10 years, body mass index (BMI, kg/m²) between 25 and 40, and taking 2 or fewer glucose-lowering medication per day. Exclusion criteria were HbA1c level greater than 9%, insulin-dependence, or presence of complications such as diabetic retinopathy, macroalbuminuria (urine albumin-creatinine ratio ≥300mg/g) or nephropathy (plasma creatinine ≥1.47 mg/dL [129.9 µmol/L]). To reduce the confounding influence of HbA1c levels at baseline, medications were standardized over a 6 weeks period prior to the study. The baseline patients characteristics, by groups, are shown in Supplementary Table 1.

All eligible patients received lifestyle advice and standard care, such as education in type 2 diabetes and medical counseling, every third month. For lifestyle group, patients additionally received an intensive lifestyle intervention, including resistance training, aerobic training, antidiabetic diet, and increased sleep duration, which has been described in detail previously (Ried-Larsen et al., 2015). All exercise sessions were supervised in the first four months, and supervision was gradually reduced thereafter. In addition, patients were encouraged to do exercise in their leisure time (≥10000 steps per day). Further details about intervention can be found in our previous study (Ried-Larsen et al., 2015). Supplementary Table 2 summarizes the patients characteristics alteration at the end of 12-month study in both groups.

At each medical counseling, every 3 months, fecal samples were collected. Samples were stored at -80°C immediately in the clinical center. A total of 413 fecal samples were
collected from 97 patients; 391 were successfully sequenced (more than 2000 sequences) (Caporaso et al., 2011) and from 86 patients with at least 3 samples from the 5 time points. The analysis of gut microbiota was performed according to the intention-to-treat principle. Although some patients did not adhere to the protocol such as low completion of exercise (less than 70% of completion), we included samples from such patients regardless of noncompliance.

Randomization and masking
Patients were randomized to either standard care group or lifestyle group in a ratio of 2:1 and stratified by sex. Random number sequence was generated by an independent statistician with computer and was given to an external data manager who was not involved in the study procedures. Patients were randomly allocated to groups by the data manager according to consecutive numbers referring to each patient. Due to the nature of the study, it is not possible to blind patients or study nurses to their group allocation, however, nurses had no role in analyzing or interpreting the data. All test personnel and adjudicators of outcomes were blinded.

Outcomes
The primary outcomes were the change of hemoglobin A1c (HbA1c) level and reduction of glucose-lowering medication use from baseline to 12 months, which was reported previously (Johansen et al., 2017). The secondary outcome, reported in the present study, was the assessment of gut microbiota during this intervention period.

DNA extraction, sequencing, and bioinformatics analysis
The total microbial DNA was extracted using the PowerMag® Soil DNA Isolation Kit on the EpMotion® 5075vt automated pipetting system (Eppendorf). The V3-V4 region of 16S rRNA gene was amplified by a two-step PCR procedure using the modified broad range primers Uni341F (5’- CCTAYGGGRBGCASCAG-3’) and Uni806R (5’-GGACTACHVGGGTWTCTAAT-3’) (Takai and Horikoshi, 2000; Klindworth et al., 2013). The amplified products were purified with Agencourt AMPure XP Beads (Beckman Coulter Genomics, MA, USA), normalized with SequaPrep™ Normalization Plate (96) Kit (Invitrogen). The concentration of pooled library was then determined using the Quant iT™ High-Sensitivity DNA Assay Kit (Life Technologies). Paired-end sequencing was performed on the Illumina MiSeq System (Illumina Inc., CA, USA) with 5.0% PhiX as an
internal control. All reagents used were from the MiSeq Reagent Kits v2 (Illumina Inc., CA, USA).

Adaptors and sequencing primers of raw FASTQ files were removed using “cutadapt” (version 1.15) (Martin, 2015). Trimmed reads were analyzed with a modified DADA2 pipeline on QIIME2 (version qiime2-2018.2) where the default overlap length of forward and reverse reads was decreased to six nucleotides (Caporaso et al., 2010; Callahan et al., 2016; Bolyen et al., 2018). Eight nucleotides were removed at the 5’ end of both forward and reverse reads to keep good sequencing quality of sequences at the denoising step and reads were not truncated by length. Other parameters in DADA2 were in default. Taxonomy was assigned against the Silva database (release 132) of 99% operational taxonomic units (OTUs) (Quast et al., 2013).

**Statistical analysis**

Alpha diversity (observed richness, Chao 1, Shannon, inverse Simpson) was assessed with R-package “phyloseq” (McMurdie and Holmes, 2013). The comparison of alpha diversity was performed with a linear mixed effect model with R-package “lmerTest” (when data followed normal distribution) (Kuznetsova et al., 2017) and a non-parametric statistical method “nparLD” with the F1-LD-F1 design (when data did not follow normal distribution) (Noguchi et al., 2012). For beta diversity, weighted and unweighted UniFrac distance were produced with function “diversity beta-phylogenetic” in QIIME2 (Bolyen et al., 2018), tested with permutational multivariate analysis of variance with adonis (R-package “vegan”) (Anderson, 2001; Lozupone and Knight, 2005; Caporaso et al., 2010; Oksanen et al., 2017). The enzyme-catalyzed reaction (EC number), ortholog (KEGG Orthology), and metabolic pathways of gut microbial communities were predicted with phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) (Ye and Doak, 2009; Langille et al., 2013). The assessment of taxa development, change in functional composition and the abundance of metabolic pathways over time was performed in the same way as for alpha diversity. Taxa, enzyme-catalyzed reactions (EC number), orthologous (KEGG Orthology), and pathways were removed before further analysis when their relative abundances were lower than 0.01%. We applied Benjamini-Hochberg correction to control false discovery rate (FDR) for multiple testing (Benjamini and Hochberg, 1995). The correlations between clinical parameters (BMI and fat percentage) and alpha diversity were assessed with linear mixed-effects regression, time was included as a covariate and repeated measurement was adjusted (function “lmer”
in R-package “lmerTest”) (Kuznetsova et al., 2017). The Random Forest models used for treatment classification were built with R-package “randomForest” (Liaw and Wiener, 2002) with 5000 trees and default value of “mtry”. The significance level was set at p<0.05 or FDR<0.05. Most plots were generated with R-package “ggplot2” (Wickham, 2016).

**Governance**

This study was performed in Region Zealand and the Capital Region of Denmark from April 2015 to August 2016. This study was approved by the Scientific Ethical Committee at the Capital Region of Denmark. All patients provided oral and written informed consent.

**Results**

**Compositional development of gut microbiota over time**

To assess the developing trend of gut microbiota between groups during the intervention period, we evaluated the alpha diversity (observed richness and Shannon diversity) in the gut microbiota of patients. From baseline (M₀) to month 12 (M₁₂), richness increased 12.3% in the lifestyle group (124.2 ± 37.3 [mean ± SD], M₀ vs. 139.5 ± 45.4, M₁₂) and 31.1% for standard care group (108.8 ± 36.4, M₀ vs. 142.6 ± 44.8, M₁₂) (Fig. 1). Shannon diversity similarly increase in the lifestyle group increased 9.5% (3.41 ± 0.48, M₀ vs. 3.73 ± 0.60, M₁₂) and standard care group increased 19.3% (3.18 ± 0.52, M₀ vs. 3.80 ± 0.56, M₁₂). Two indices increased profoundly from 0 to 3 months for both groups. To compare the development of alpha diversity, we fitted non-parametric model to assess the change of richness and Shannon diversity over time. It showed that time was a strong influencing factor (p<0.001 for both richness and Shannon), but the development of two alpha indices between groups did not differ significantly (p=0.52, richness; p=0.38, Shannon; interaction between time and treatment).

Next, we correlated alpha diversity indices with clinical parameters for patients in both groups. In the lifestyle group, the observed richness and Chao 1 diversity were negatively correlated with BMI (p=0.009 and p=0.004, respectively) and fat percentage (p=0.004 and p=0.003, respectively; Fig. 2). However, Shannon and inverse Simpson diversity did not represent significant associations. In contrast, for standard care group, alpha diversity and clinical parameters displayed weaker correlations where only observed richness and BMI had a significantly positive correlation (p=0.037). But, two groups differed in correlation trend in alpha diversity indices and body compositional parameters (except inverse Simpson diversity) (p<0.05, interaction between alpha diversity and group).
Fig. 1. Alpha diversity over time between groups. Distribution of observed richness and Shannon diversity for lifestyle (red) and standard care groups (blue). Each dot refers to a sample. Lines across dots are the mean of alpha diversity values in corresponding groups at each time point and the standard deviation are shown by error bars accordingly.

Fig. 2. The correlations of alpha diversity with BMI and fat percentage between groups. The lines indicate the mixed linear regression between clinical parameters and alpha diversity. Significance “Yes” indicates a line whose slope is significantly different from zero, and vice versa for significance “No”. Values for BMI and fat percentage are in the unit of kg/m² and %, respectively.
To describe the difference in the overall structure of gut microbiota at each time point between groups, we compared Bray-Curtis dissimilarity, weighted and unweighted UniFrac distance. Samples from two groups were overlapped when visualized with principal coordinates analysis (PCoA) at all time points (Fig. 3). Variance attributed to treatment (lifestyle intervention or standard care) was significant at 3 months for Bray-Curtis dissimilarity (p=0.039) and weighted UniFrac distance (p=0.017), but no longer significant after p value correction (p=0.196 and p=0.196 for both distance metrics) (Supplementary Table 3). To assess the time effect on gut microbiota, we compared the beta diversity of both groups between M_{12} and M_{0}. It showed that the structure of gut microbial communities in both groups changed significantly (Fig. S1, Supplementary Table 4). Treatment represented a small but significant proportion of variance when incorporating all samples (not stratified by time points) (R^2=0.0086, p<0.001; R^2=0.0125, p<0.001; R^2=0.0057, p<0.001; for Bray-Curtis dissimilarity, weighted and unweighted UniFrac distance and, respectively; Fig. S2). But the interaction between time and treatment was not significant (p=0.99, Bray-Curtis; p=0.97, weighted UniFrac distance; p=1, unweighted UniFrac distance; interaction between time and treatment).

Fig. 3. The distribution of samples between lifestyle (red) and standard care (blue) groups, assessed with Bray-Curtis dissimilarity, weighted and unweighted UniFrac distance, visualized by principal coordinates analysis (PCoA), with ellipses encircling 75% of samples from each group.
To depict the taxonomic composition of gut microbiota in two groups, we visualized the top 10 most abundant phyla over time (Fig. 4). The gut microbiota of patients was dominated by the phylum Firmicutes, which accounted for 72.1% of sequences, followed by Bacteroidetes, Verrucomicrobia, Proteobacteria and Actinobacteria, which had a combined relative abundance of 98.7% of all sequences. To assess the development of gut microbial communities in two groups over time, we analyzed the change of taxa abundance at all taxonomic levels. Time showed a strong effect on the development of taxa that 2 phyla, 5 classes, 11 orders, 16 families, 35 genera, 45 ASVs developed significantly (data not shown). At phylum level, Bacteroidetes increased over time (FDR<0.001), while Actinobacteria declined from M0 to M12 (FDR<0.001) (Fig. S3). Despite the strong time effect on gut microbiota, we did not find any taxa with significantly different changes between the groups (FDR>0.05, interaction between time and treatment).

![Fig. 4. Taxonomic composition of gut microbiota in the lifestyle intervention and standard care groups. Bar-plot showing the top 10 most abundant phyla. The reset of phyla was merged as “Others”.

To further elucidate the treatment effect, we applied the Random Forest, a machine learning algorithm, to identify the treatment for a patient purely based on the gut microbiota. First, the algorithm was applied at each time point and samples from standard care group were always identified as lifestyle intervention group (error rate 100%). Next,
the error rate to differentiate two groups was lowered when including samples from all time points, but still 65% of standard care samples in standard care were incorrectly identified as lifestyle group.

**Functional development of gut microbiota over time**
To assess if the functional composition of microbial communities in the gut shifted during the 12-month follow up, the metagenome were predicted with 16S rRNA gene amplicon using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2). The abundance of EC numbers and orthologs were tested against treatment, time and their interaction. Time was the strongest influencing factor with EC numbers were changing significantly over time. 27 EC numbers showed different relative abundances between groups. However, none of these EC numbers developed differently between groups over time (FDR>0.05, interaction between time and treatment). Similarly, 1027 orthologs changed significantly over time and 123 differed in relative abundance between groups, but none of them developed differently between groups (FDR>0.05, interaction between time and treatment).

We further grouped enzyme-catalyzed reactions into metabolic pathways against MetaCyc database with MinPath and a total of 408 pathways were retrieved, of which 219 pathways were changing significantly over time, but none of these pathways differed in their relative abundance between groups (FDR>0.05, treatment effect), or changed differently between groups over time (FDR>0.05, interaction between time and treatment).

**Discussion**
To investigate possible side effects of glucose-lowering medication (first-line biguanid [tablet Metformin] and second-line GLP-1-analogue liraglutide [Victoza]) use on gut microbiota, we performed a randomized, assessor-blinded, single-center clinical trial in T2D individuals allocated to either medication or additionally having intensive lifestyle intervention. After a 12-month trial, despite the higher increase in physical fitness in the lifestyle intervention group than in the standard care group (Supplementary Table 2) (Johansen et al., 2017), the gut microbiota of patients between groups did not develop differently.

In the present study, a 12-month of lifestyle intervention resulted in a massive shift in gut microbial alpha diversity (Fig. 1). We observed 12.3% and 9.5% of increase in richness and Shannon diversity, respectively. It concurs with the findings that diet intervention and
exercise were able to increase the gut microbial diversity accompanied with improved physiological fitness (Cotillard et al., 2013; Clarke et al., 2014). The increase of gut microbiota diversity in our data reflects patients were physiologically increasingly healthy. Because microbial diversity was emerging as a biomarker of health status that a low gut bacterial richness was associated with metabolic abnormalities and negatively correlated with metabolic risk markers (Shanahan, 2010; Le Chatelier et al., 2013; Brahe et al., 2015).

In fact, the most significant increase of alpha diversity happened during the first 3 months in our data, the richness stabilized thereafter. Such a tremendous increase in alpha diversity could be attributed to the abrupt interruption of lifestyle within the first 3 months when the physical exercise was strictly supervised and healthy diet was recommended. However, we also observed a similar trend of alpha diversity in the standard care group compared to lifestyle intervention group (Fig. 1), which is to some extent beyond our expectation. Although patients in standard care group did not represent significantly improved their physical fitness as patients in the lifestyle intervention group (Supplementary Table 2), they did have increased observed richness (31.1%) and Shannon diversity (19.3%). Considering the glucose-lowering medication especially metformin was likely the main driving force of alpha diversity for patients in the standard care group, it indicates that medication drives the gut microbiota to a similar direction as the effect of lifestyle intervention in terms of microbial alpha diversity. In human, Simpson diversity has been reported to be increased after 12 weeks of metformin treatment (Tong et al., 2018), and increased gut microbiome richness has been observed in individuals with T2D when administrated metformin, even though the effect was not strong enough to be significant (Forslund et al., 2015).

For beta diversity, the influence of treatment was only detected at month 3 and 9 (Fig. 3, Supplementary Table 3). To investigate if two groups developed differently in beta diversity, one could assess the interaction between time and treatment. However, the effect of time on gut microbiota was treatment independent. The absence of interaction between time and treatment was further confirmed by assessing taxa at all taxonomic levels and the analysis of functional potential (enzyme-catalyzed reactions, orthologs, and metabolic pathways), where no differences in developing trend were observed between groups. However, some weak differences were observed between groups. For instance, variance attributed to treatment was not strong to be significant when stratified by time, but treatment significantly explained a proportion of variance when accumulating variance from all time points by incorporating all samples (Fig. S2). In addition, although the
correlations between body compositional parameters and alpha diversity were not always significant, the correlation trend differed significantly between two groups. This divergence was reasonable when considering that alpha diversity increased in both groups but not proportional to the change of body compositional parameters (Supplementary Table 2). Of interest, we found that alpha indices that having a different balance between rareness and evenness (Morris et al., 2014) correlated differently with body compositional parameters. Observed richness and Chao1 were more likely to be correlated with clinical parameters compared to Shannon and inverse Simpson. It supported the suggestion that indices sensitive to rareness are more liable to detect the effects of external factors on diversity (Heino et al., 2008; Magurran and Dornelas, 2010).

We did not observe clear distinction in the gut microbial development between groups, but a strong effect of time based on alpha and beta diversity, comparison of taxa abundance, and functional analysis. Numerous taxa changed significantly over time. For instance, at the phylum level, the relative abundance of Bacteroidetes increased over time in both groups and Actinobacteria decreased (Fig. S3). Bacteroidetes and Firmicutes are two phyla well known for the association with obesity. Ley et al. (2005) found a 50% reduction of Bacteroidetes along with a corresponding degree of increase in Firmicutes was observed in the distal intestine of obese mice compared to lean mice. Turnbaugh et al. (2006) further investigated this using shot-gun metagenomic sequencing and observed an increased ratio of Firmicutes to Bacteroidetes and enhanced capacity to harvest energy from the diet in obese mice compared to lean littermates (Turnbaugh et al., 2006). A similar correlation between the ratio of Bacteroidetes to Firmicutes and obesity was also observed in human (Ley et al., 2006), and Bacteroidetes alone seemed to be more tightly related with obesity than Firmicutes and accounted for 42% of the lean-enriched genes (Turnbaugh et al., 2009). Actinobacteria is another phylum considered as obese related from where 75% of the obesity-enriched genes were derived (Turnbaugh et al., 2009). Collectively, the phylum-wide increase of Bacteroidetes and decrease of Actinobacteria in our data imply that patients in both groups seemed increasingly healthy (Supplementary Table 2). Although two groups achieved different degrees of fitness, such deviation was not detected based on the gut microbiota.

In conclusion, our study revealed that improved health impacted the gut microbiota of diabetic patients similarly when driven by a lifestyle intervention or glucose-lowering medication. Gut microbiota of patients in both groups changed massively during the follow up, but the development over time did not differ significantly. Considering two groups
achieved significantly different physical fitness during the study period, we speculate that gut microbiota were not fundamental for the improvements of fitness.

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Reference:
microbial diversity. Gut 63: 1913–20


Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O’Hara RB,


Supplementary material

**Fig. S1.** The distribution of samples between month 0 (red) and 12 (blue), assessed with Bray-Curtis dissimilarity, weighted and unweighted UniFrac distance, visualized by principal coordinates analysis (PCoA), with ellipses encircling 75% of samples from each group.

**Fig. S2.** Sample distribution between lifestyle (red) and standard care (blue) groups, assessed with Bray-Curtis dissimilarity, weighted and unweighted UniFrac distance, visualized by principal coordinates analysis (PCoA), with ellipses encircling 75% of samples from each group.
Fig. S3. The top 5 most abundant phyla in the gut microbiota over time between lifestyle intervention and standard care groups. The relative abundance is log_{10} scaled for visualization. The reset of phyla was merged as “Others”. Significance refers to the change of relative abundance of a phylum over time was significant (FDR<0.05, time effect, solid lines) or not significant (FDR>0.05, dashed lines). Lines are coloured according to the respective phylum.

Supplementary Table 1. Baseline characteristics of patients in lifestyle intervention and standard care groups.

<table>
<thead>
<tr>
<th></th>
<th>Lifestyle intervention (n=60)</th>
<th>Standard care (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>53.2 (9.1)</td>
<td>57.4 (8.1)</td>
</tr>
<tr>
<td><strong>Male No. (%)</strong></td>
<td>32 (53.3)</td>
<td>13 (50)</td>
</tr>
<tr>
<td><strong>Glycemic control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin A1c, mmol/mol</td>
<td>48.8 (9.0)</td>
<td>50.5 (9.8)</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl</td>
<td>7.61 (1.69)</td>
<td>8.15 (1.86)</td>
</tr>
<tr>
<td>Fasting insulin, ul U/ml</td>
<td>123.8 (54.7)</td>
<td>125.1 (59.7)</td>
</tr>
<tr>
<td>2-h glucose, mg/dl</td>
<td>15.2 (4.0)</td>
<td>16.3 (4.0)</td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-density lipoprotein (LDL), mg/dl</td>
<td>2.44 (0.78)</td>
<td>2.26 (0.91)</td>
</tr>
<tr>
<td>High-density lipoprotein (HDL), mg/dl</td>
<td>1.22 (0.35)</td>
<td>1.29 (0.38)</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>1.51 (0.68)</td>
<td>1.62 (1.03)</td>
</tr>
<tr>
<td><strong>Glucose-lowering medication No. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Biguanide</td>
<td>48 (80.0)</td>
<td>21 (80.7)</td>
</tr>
<tr>
<td>Biguanide and GLP-1 analogue</td>
<td>11 (18.3)</td>
<td>5 (19.2)</td>
</tr>
<tr>
<td>Biguanide, GLP-1 analogue, and insulin</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Glucose-lowering medication score

<table>
<thead>
<tr>
<th></th>
<th>Lifestyle intervention (n=60)</th>
<th>Standard care (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid-lowering medication No. (%)</td>
<td>2.86 (1.32)</td>
<td>2.92 (1.29)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>12 (20.0)</td>
<td>4 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>48 (80.0)</td>
<td>22 (84.6)</td>
<td></td>
</tr>
</tbody>
</table>

Lipid-lowering medication score

<table>
<thead>
<tr>
<th></th>
<th>Lifestyle intervention (n=60)</th>
<th>Standard care (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body composition</td>
<td>2.95 (1.98)</td>
<td>3.15 (1.80)</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>54.9 (7.1)</td>
<td>55.4 (7.7)</td>
<td></td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>95.6 (14.2)</td>
<td>95.8 (15.1)</td>
<td></td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>35.7 (9.0)</td>
<td>35.9 (11.1)</td>
<td></td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>55.9 (10.3)</td>
<td>56.0 (9.2)</td>
<td></td>
</tr>
<tr>
<td>Gynoid fat mass, kg</td>
<td>5.41 (1.53)</td>
<td>5.32 (2.10)</td>
<td></td>
</tr>
<tr>
<td>Android fat mass, kg</td>
<td>4.01 (1.14)</td>
<td>4.19 (1.32)</td>
<td></td>
</tr>
<tr>
<td>Fat percentage, %</td>
<td>38.9 (7.7)</td>
<td>38.6 (8.9)</td>
<td></td>
</tr>
<tr>
<td>Fat free mass, kg</td>
<td>59.2 (10.7)</td>
<td>59.3 (9.7)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean (standard deviation) or No. (percentage). Abbreviation: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared). Medication score ranges: glucose-lowering medication, 0 to 7; blood pressure-lowering medication, 0 to 8; and lipid-lowering medication, 0 to 6. A higher medication score indicates a more-intensive pharmacological treatment.

Supplementary Table 2. The alteration of characteristics of patients after 12-month follow up in lifestyle intervention and standard care groups.

<table>
<thead>
<tr>
<th></th>
<th>Lifestyle intervention (n=60)</th>
<th>Standard care (n=26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemic control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin A1c, mmol/mol</td>
<td>-3.367 (7.50)</td>
<td>-2.48 (9.17)</td>
<td>0.672</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl</td>
<td>-0.83 (1.58)</td>
<td>-0.94 (2.01)</td>
<td>0.801</td>
</tr>
<tr>
<td>Fasting insulin, ul U/ml</td>
<td>-36.7 (50.4)</td>
<td>-19.1 (45.4)</td>
<td>0.13</td>
</tr>
<tr>
<td>2-h glucose, mg/dl</td>
<td>-2.64 (2.98)</td>
<td>-1.41 (4.00)</td>
<td>0.175</td>
</tr>
<tr>
<td>Lipid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-density lipoprotein (LDL), mg/dl</td>
<td>0.32 (0.82)</td>
<td>0.28 (1.22)</td>
<td>0.884</td>
</tr>
<tr>
<td>High-density lipoprotein (HDL), mg/dl</td>
<td>0.22 (0.27)</td>
<td>0.17 (0.29)</td>
<td>0.487</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>-0.21 (0.71)</td>
<td>-0.15 (0.73)</td>
<td>0.766</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of patients with reduction in glucose-lowering medication No. (%)</td>
<td>51 (85)</td>
<td>10 (39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose-lowering medication score</td>
<td>-1.63 (1.57)</td>
<td>0.48 (1.92)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proportion of patients with reduction in lipid-lowering medication No. (%)</td>
<td>34 (57)</td>
<td>13 (50)</td>
<td>0.751</td>
</tr>
<tr>
<td>Lipid-lowering medication score</td>
<td>-0.14 (1.95)</td>
<td>0.04 (1.99)</td>
<td>0.709</td>
</tr>
<tr>
<td>Body composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>-3.55 (4.20)</td>
<td>-1.41 (3.95)</td>
<td>0.031</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>-6.20 (7.34)</td>
<td>-2.43 (6.76)</td>
<td>0.027</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>-6.28 (6.47)</td>
<td>-1.51 (4.68)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>0.73 (2.35)</td>
<td>-1.14 (2.73)</td>
<td>0.005</td>
</tr>
<tr>
<td>Gynoid fat mass, kg</td>
<td>-0.85 (0.96)</td>
<td>-0.20 (0.58)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Data are presented as mean (standard deviation) or No. (percentage). p values are from two-sided two-sample t-test. Abbreviation: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared). Medication score ranges: glucose-lowering medication, 0 to 7; blood pressure-lowering medication, 0 to 8; and lipid-lowering medication, 0 to 6. A higher medication score indicates a more-intensive pharmacological treatment.

Supplementary Table 3. Comparisons of beta diversity between lifestyle intervention and standard care groups.

<table>
<thead>
<tr>
<th>Distance</th>
<th>Time (months)</th>
<th>Axis1</th>
<th>Axis2</th>
<th>p value</th>
<th>p value adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bray-Curtis</td>
<td>0</td>
<td>8.7%</td>
<td>8.1%</td>
<td>0.500</td>
<td>0.683</td>
</tr>
<tr>
<td>weighted UniFrac</td>
<td>0</td>
<td>28.6%</td>
<td>15.1%</td>
<td>0.598</td>
<td>0.743</td>
</tr>
<tr>
<td>Unweighted UniFrac</td>
<td>0</td>
<td>11.9%</td>
<td>6.9%</td>
<td>0.448</td>
<td>0.683</td>
</tr>
<tr>
<td>Bray-Curtis</td>
<td>3</td>
<td>9.6%</td>
<td>7.7%</td>
<td>0.039</td>
<td>0.196</td>
</tr>
<tr>
<td>weighted UniFrac</td>
<td>3</td>
<td>23%</td>
<td>16.2%</td>
<td>0.017</td>
<td>0.196</td>
</tr>
<tr>
<td>unweighted UniFrac</td>
<td>3</td>
<td>9.3%</td>
<td>4.4%</td>
<td>0.173</td>
<td>0.380</td>
</tr>
<tr>
<td>Bray-Curtis</td>
<td>9</td>
<td>10.5%</td>
<td>7.6%</td>
<td>0.034</td>
<td>0.196</td>
</tr>
<tr>
<td>weighted UniFrac</td>
<td>9</td>
<td>19.7%</td>
<td>15.7%</td>
<td>0.062</td>
<td>0.256</td>
</tr>
<tr>
<td>unweighted UniFrac</td>
<td>9</td>
<td>10.1%</td>
<td>4.9%</td>
<td>0.644</td>
<td>0.743</td>
</tr>
<tr>
<td>Bray-Curtis</td>
<td>6</td>
<td>11.6%</td>
<td>9.6%</td>
<td>0.095</td>
<td>0.284</td>
</tr>
<tr>
<td>weighted UniFrac</td>
<td>6</td>
<td>22.8%</td>
<td>20.6%</td>
<td>0.185</td>
<td>0.380</td>
</tr>
<tr>
<td>unweighted UniFrac</td>
<td>6</td>
<td>9.9%</td>
<td>5.1%</td>
<td>0.483</td>
<td>0.683</td>
</tr>
<tr>
<td>Bray-Curtis</td>
<td>12</td>
<td>8.1%</td>
<td>7.3%</td>
<td>0.721</td>
<td>0.773</td>
</tr>
<tr>
<td>weighted UniFrac</td>
<td>12</td>
<td>20.8%</td>
<td>15.5%</td>
<td>0.203</td>
<td>0.380</td>
</tr>
<tr>
<td>unweighted UniFrac</td>
<td>12</td>
<td>11.5%</td>
<td>8.8%</td>
<td>0.817</td>
<td>0.817</td>
</tr>
</tbody>
</table>

The values shown in “Axis 1” and “Axis 2” are the variance explained when visualized with principal coordinates analysis. The p values are from the permutational multivariate analysis of variance. Adjusted p values are from the Benjamini-Hochberg correction.
Supplementary Table 4. The comparisons of beta diversity between baseline (M₀) and month 12 (M₁₂) stratified by groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Distance</th>
<th>Axis1</th>
<th>Axis2</th>
<th>p value</th>
<th>p value adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lifestyle</td>
<td>Bray-Curtis</td>
<td>8.0%</td>
<td>5.9%</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lifestyle</td>
<td>weighted UniFrac</td>
<td>23.0%</td>
<td>14.2%</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lifestyle</td>
<td>unweighted UniFrac</td>
<td>10.8%</td>
<td>6.2%</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Standard care</td>
<td>Bray-Curtis</td>
<td>10.5%</td>
<td>9.4%</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Standard care</td>
<td>weighted UniFrac</td>
<td>27.4%</td>
<td>13.6%</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Standard care</td>
<td>unweighted UniFrac</td>
<td>13.3%</td>
<td>8.8%</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The values shown in “Axis 1” and “Axis 2” are the variance explained when visualized with principal coordinates analysis in corresponding axes. The p values are from the permutational multivariate analysis of variance. Adjusted p values are from the Benjamini-Hochberg correction.
Manuscript 3
A glance into spatial and individual variability of rhizosphere microbiota

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¹Department of Biology, Section of Microbiology, University of Copenhagen, Universitetsparken 15, bldg. 1, DK-2100, Copenhagen, Denmark.
²INRA, UMR 1347 Agroécologie, Dijon, France

Abstract

Managing plant-host root microbial communities to steer crop productivity and health stands as one of the most important challenges for future sustainable agriculture. This often relies on the molecular characterization of the root microbiota using a homogenized rhizosphere sample of the entire root system of a given host-plant. While this traditional procedure enabled reproducibility between studies and significant advances in our understanding of this complex ecosystem, concerns have been raised about the necessity to go at a finer scale to better decipher plant-microbes interactions. Here we show the importance of a smaller sampling scale to better depict the rhizosphere microbiota. We applied a three-generation experiment with the model plant Brachypodium distachyon in culture chamber, where each rhizosphere sample from a plant was collected either by i) selecting three independent roots that were further cut into three fragments (root tip, middle section, rear section; called “subscale rhizosphere”) and ii) all the remaining roots (standard homogenized rhizosphere). We further investigated the importance of sampling scale by testing its relevance to detect effects of a microbial inoculation applied on plants or not. At subscale, we showed that rhizosphere microbiota along root axis was heterogeneous in alpha diversity, beta diversity, taxonomic composition, but also in the response to inoculation. Conversely, traditional sampling reduced the variance between replicates, giving higher reproducibility at the expense of not fully seeing the influence of inoculation on rhizosphere microbiota. Our results bring a significant contribution to the field inoculation, revealing the importance of traditional sampling for higher
reproducibility, while finer subscale approach enables the detection of microbial inoculation effects, which are yet often hard to observe and understand in many cases, likely due to inappropriate sampling strategy.

1. Introduction

There is a high demand to increase plant productivity to meet the growing human population. A sustainable way to achieve this is utilizing plant associated microbiota, especially the microbiota surrounding plant roots, a zone termed rhizosphere (Hiltner, 1904). Rhizosphere is a hotspot of biochemical reactions that provide essential nutrients for plant growth (Kuzyakov and Blagodatskaya, 2015). To successfully utilize rhizosphere microbiota as a way to increase crop yield, it is crucial to understand its community structure and key factors influencing it.

The most popular way to investigate root-microbiota is via a traditional procedure where a homogeneous sample of the entire rhizosphere of the host-plant is taken (Barillot et al., 2013). From this traditional sampling, significant knowledge was gained on distinct characteristics of these microbial communities living in the rhizosphere, as opposed to microbes dwelling in the bulk soil. These differences are instigated by the presence of plant roots via biological, biochemical, chemical and physical processes (Hinsinger et al., 2005). While traditional sampling revealed useful in many instances, however, it destroys and neglects the fine-scale microbial community structure established in response to crucial influencing factors, which is key to understand plant-microbe interaction.

At the scale of a single root, high heterogeneity of biochemical and chemical processes along root axis was observed due to the variation of pH, water content, redox potential, enzyme activities (Hinsinger et al., 2009; Carminati, 2013; Razavi et al., 2016; Kreuzeder et al., 2018). Despite being poorly explored due to technological limitations (Philippot et al., 2013), such spatial heterogeneities are known to inevitably lead to uneven distribution of root microbiota, thus calling for more focus on this bottleneck. A few studies attempted to fill the knowledge gap in a range of plants, such as oat (DeAngelis et al., 2009), wheat (Semenov et al., 1999; Watt et al., 2003; Watt et al., 2006), tomato (Chin-A-Woeng et al., 1997; Gamalero et al., 2004) and cucumber (Folman et al., 2001). However, these studies were limited either by the profiling method used or narrowed to specific strains, making it difficult to infer general principles.

Inoculation of plant growth promoting rhizobacteria (PGPR) (such as nitrogen-fixing and phosphorous solubilizing bacteria) (Bhattacharyya and Jha, 2012) or artificially selected
microbial consortia (Panke-Buissé et al., 2014) to plant roots is a promising way to alter or improve the host physiology. But securing the survival and efficiency of inoculants is challenging (Herrmann and Lesueur, 2013), as it highly depends on the ability to establish and maintain on the roots as they grow and age (Dutta and Podile, 2010). Crucial knowledge of inoculant survival is currently lacking, thus hampering the success of field trials and subsequent applications. Therefore, it is important to probe their microsite colonization patterns after inoculation, which cannot be achieved through mere traditional rhizosphere collection approaches.

Just as for traditional rhizosphere sampling methods, the use of biological replicates is the rule for reproducibility, where identical seedlings are grown in different pots and exposed to the same environmental conditions. Nevertheless, inter-replicate variation, here defined as differences of rhizosphere microbiota amongst individuals within the same treatment group, is always expected due to the individual variation (Briggs and Walters, 2016). Indeed, the plant individual variation of rhizosphere microbiota might arise from variation in root development, environmental heterogeneity, host-plant physiology, technical variations brought by experimenters, and seed-borne microbial transmission (Puente et al., 2009; Hardoim et al., 2012; Johnston-Monje et al., 2016). Furthermore, local variations may arise from the so-called “priority effects” instigated by microbial pioneers via influencing of subsequent recruitment, resulting from stochastic colonization patterns (Fukami, 2015). The extent of variance partition associated with such individual plant variation is not accessible via traditional rhizosphere sampling but may be achieved by sampling at a smaller scale.

In this study, we used Brachypodium distachyon, as a model plant to follow rhizosphere microbiota in multiple ways including the spatial distribution, the influence of individual variation and effect of microbial inoculation. We hypothesized that fine subscale sampling will result in a higher resolution to detect significant effects that might be missed by traditional approaches. This study represents a considerable contribution to the current knowledge on root microbiota, revealing the joint importance of both methods for better understanding of community assembly patterns depending on research questions and aims.

2. Material and methods

2.1 Soil and plant growth conditions

A poor sandy soil classified as cambisol with moorland was used (organic carbon: 14.7 g kg\(^{-1}\); total nitrogen: 1.19 g kg\(^{-1}\); pH: 5.22; clay: 6.9%, loam: 19.0%, sand: 74.1%, origin:
CEREEP, Saint-Pierre-Lès-Nemours, France). Soil was sampled in February 2016 avoiding plant material and brought back to INRA (Dijon) the same day. The soil was laid down and dried in a tempered room for a week, followed by machine-assisted sieving at 2 mm. The soil was then stored in a sealed GeoBox at the INRA greenhouse facility. Before each generation, the soil was autoclaved once in bags (115°C, 45 min) and allowed to rest for 48h before use. 350 g of dried autoclaved soil were transferred in small pots and placed in watering cups with 80ml osmosis water to allow bottom irrigation up to 80-90% of the water holding capacity (WHC).

*Brachypodium distachyon* seeds used in this study are coming from a stable genetic breed grown at the INRA de Versailles. Seeds were placed into parafilm-sealed transparent boxes with humidified blotting paper and left a 4°C in the dark for 24h for vernalization. Afterwards, boxes were placed in a germinator for 48h in the dark at 18°C, followed by full-light at 20°C for four days (ARALAB, FITOCLIMA600 PL/PLH). Water was provided to maintain maximum humidity in boxes. Obtained seedlings were transplanted into the prepared soil pots described above and place immediately into a climatic chamber (ARALAB, WALK-IN EH, 22°C, 12h light, 70% air humidity). Pots were bottom-irrigated manually twice a week, including a regularization every Friday via weighing to ensure that humidity is maintained at 70-90% WHC. Pots were manually randomized every Wednesday to avoid border effects in the chamber.

### 2.2 Study design and sampling

The present study is part of a larger research initiative conducted in multiple generations using plant *Brachypodium distachyon*. In each generation “N”, 20 plants were inoculated with an aqueous mixture obtained from the rhizosphere soil of the three greenness plants in the previous generation “N-1”, as adapted from previous procedures (Panke-Buisse et al, 2015). The plant phenotyping for leave greenness determination was done via image analysis at the 4PMI platform (For Plant and Microbe Interactions, INRA Centre Dijon, France, see acknowledgments). The present study was performed on three consecutive generations. At each generation, the rhizosphere from the three greenest plants was used to inoculate the 20 plants on the next generation. Amongst the 17 remaining plants after removing three plants for inoculation, five were selected based on leave greenness homogeneity as our inoculated group (Fig. 1A). In addition, five growth control plants without any inoculations were considered as our non-inoculated control group to account
for environmental microbial colonization coming from the experimental design (air, soil, and water).

<table>
<thead>
<tr>
<th>Generation</th>
<th>Inoculum</th>
<th>Inoculated group</th>
<th>Non-inoculated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0</td>
<td>3 out of 20 plants, for inoculation G1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>3 out of 20 plants, for inoculation G2</td>
<td>5 out of 20 plants</td>
<td>5 growth controls</td>
</tr>
<tr>
<td>G2</td>
<td>3 out of 20 plants, for inoculation G3</td>
<td>5 out of 20 plants</td>
<td>5 growth controls</td>
</tr>
<tr>
<td>G3</td>
<td></td>
<td>5 out of 20 plants</td>
<td>5 growth controls</td>
</tr>
</tbody>
</table>

Fig. 1. Flowchart showing the study design (A) and sampling process (B). 20 plants were grown in the inoculation lineage every generation, three of which were used for inoculation, and five of which were considered as our inoculated group. Five growth control plants in each generation were considered as our non-inoculated group. Sample types “T”, “M”, “R” and “H” refer to the tip, middle, rear part of a root, and homogenized roots, respectively.

![Flowchart](image)

To collect the rhizosphere microbiota (Fig. 1B), the root system of a plant was carefully removed from the pot, loosely attached soil was gently shaken off. The remaining soil, tightly attached to the roots, was considered as the rhizosphere. Three roots in similar phenotype were selected from one plant and visible lateral roots were removed. Each root was further cut into three fragments separately and named as “T” (tip: the first 1 cm of a selected side root), “M” (middle: the next 3 cm) and “R” (rear: the remaining part for the side root fragment, ~10 cm). The remaining root system of each plant was used as a
traditional homogenized rhizosphere sample (Barillot et al., 2013), and named as “H” (homogenized). To alleviate eventual biases coming from varying fragment length, data were normalized accordingly via rarefaction curves to estimate sequencing depth (Fig. S1) and adjusted statistical methods (see 2.6 Statistical analysis). The rhizosphere soil from “T”, “M” and “R” was collected by dissolving samples in 1 ml of sterile 0.9% in a 2 ml tube NaCl and vortexed horizontally at 6000 rpm for 20 min. The rhizosphere soil of “H” sample was sampled by vortexing at 6000 rpm for 2 min, in a 50 ml tube with 5 ml of sterile 0.9% NaCl. The collected rhizosphere samples were stored at -20°C.

2.3 Inoculants and inoculation
The inoculum was made of a rhizosphere soil slurry made from three individual root systems coming from Brachypodium distachyon plants in the previous generation. Three root systems containing rhizosphere soil were pooled together, dissolved in 200 ml greenhouse tap water, vortexed at 500 rpm for 30 min with a magnetic stirrer. Right after, 50 ml of the so-obtained liquid slurry was collected for DNA extraction, 50 ml were used for glycerol stock preservation, and the remaining 100 ml were used to inoculate the next generation seedlings as follow: i) bath-exposed rapidly to the slurry (~10s) then ii) transferred immediately in the pots and inoculated with 1 ml of the slurry inside the transplantation hole and finally iii) inoculated with another 1 ml before going to the climatic chamber.

2.4 DNA extraction, sequencing, and bioinformatics analysis
The subscale sample solutions were concentrated by centrifugation at 16,000 g for 20 min, 650 µl supernatant was removed and the pellet was resuspended using FastPrep-24 at 4.0 m/s for 60 seconds. Thereafter, the rhizosphere microbial DNA of subscale and traditional samples was extracted using the DNeasy PowerSoil HTP 96 Kit (QIAGEN). For the mechanical lysis step, we increased shaking time to 15 min at 20 Hz, then followed the manufacturer’s instruction for the remaining steps. Extracted DNA was diluted with 30 µl of elution buffer. In total, 25 DNA extraction negative controls were included, by adding 200 µl of molecular grade water (Sigma-Aldrich, Merck, Germany) instead of samples. Extracted DNA was stored at -20°C.

The 16S rRNA gene fragment targeting the V3-V4 regions was amplified in two steps: first, amplification of hypervariable V3-V4 region, using the modified universal primers 341F (5’- CCTAYGGGRBGCASCAG -3’) and 806R (5’-
GGACTACHVGGGTWTCTAAT-3’) (Takai and Horikoshi, 2000; Klindworth et al., 2013); then the adaptors and sequencing primers were added to the amplicon products at the second step. The amplified products were purified with Agencourt AMPure XP Beads (Beckman Coulter Genomics, MA, USA), normalized with SequaPrep™ Normalization Plate (96) Kit (Invitrogen). The centration of the pooled library was then determined using the Quant-iT™ High-Sensitivity DNA Assay Kit (Life Technologies). Paired-end sequencing was performed on the Illumina MiSeq System (Illumina Inc., CA, USA) with 5.0% PhiX as the internal control. All reagents used were from the MiSeq Reagent Kits v2 (Illumina Inc., CA, USA). Adaptors and sequencing primers of raw FASTQ files were removed using “cutadapt” (version: 1.15) (Martin, 2011). Trimmed reads were analyzed with a modified DADA2 pipeline on QIIME2 (version: qiime2-2018.2) where the default overlap length (for merging paired reads) of forward and reverse reads was decreased to 6 nucleotides (Caporaso et al., 2010; Callahan et al., 2016). Eight nucleotides were removed at the 5’ end of both forward and reverse reads to keep a good sequencing quality at the denoising step, and reads were not truncated by length. Other parameters in DADA2 were set as default. With the algorithms of DADA2, sequences were resolved into exact sequences features, called amplicon sequence variant (ASV). Taxonomy was assigned using the Silva database (release 132) of 99% identity criteria (Quast et al., 2013).

2.5 Statistical analysis

After removing eight samples with low sequencing depth (below 2000 reads; Fig. S1) (Caporaso et al., 2011), 292 samples remained available for further analysis. To minimize the sample size effect, only ASVs that were present in at least 10 samples were kept to remove features with low occurrences. In addition, taxa having relative abundance lower than 0.01% were removed before comparing their abundance. To account for the generation effect, the alpha diversity of root sections “T”, “M” and “R” was calculated with R-package “phyloseq” (McMurdie and Holmes, 2013) and standardized as “z-scores” by subtracting the mean of “H” and then divided by the standard deviation of “H” in corresponding generation, formulated as (Diversity_{T/M/R} - mean(Diversity_{H}))/SD(Diversity_{H}). The comparisons of standardized alpha diversity were performed with two-sided two-sample t-test. Comparisons of UniFrac distances, produced with function “qiime diversity beta-phylogenetic” in QIIME2, between root sections were tested with permutational multivariate analysis of variance (PERMANOVA, adonis function, package “vegan”) (Anderson, 2001; Lozupone and Knight, 2005; Caporaso et al., 2013).
Comparisons of taxa abundance at ASV level were performed with “limma” methodology with the function “DA.lli2” in the R-package “DAtest” (Ritchie et al., 2015; Russel et al., 2018), repeated measurements were adjusted by assigning plant identity to the parameter “paired” and included generation as a covariate. At higher taxonomic levels, comparison of taxa abundance was performed with two-way ANOVA (generation effect was adjusted by including as a covariate). We applied Benjamini–Hochberg correction to control the false discovery rate (FDR) for multiple testing (Benjamini and Hochberg, 1995). The comparison of median UniFrac distance between root sections was assessed with Wilcoxon rank-sum test in R-package “stats”. Most plots were generated with R-package “ggplot2” (Wickham, 2016).

To have a general knowledge of *Brachypodium distachyon* root system, analysis regarding the heterogeneity of root sections such as alpha diversity, beta diversity, discriminant taxa, and pairwise UniFrac distance were performed only with non-inoculated plants. While both non-inoculated and inoculated plants were used to investigate the influence of inoculants on root sections.

3. Results

3.1 Compositional variation along root axis

Along the *Brachypodium* root axis, we observed increasing alpha diversity from tip to base (Fig. 2A). In detail, the standardized richness of root tip samples “T” was significantly lower than the middle root “M” and the rear root “R” fragments (p<0.001 and p<0.001, T vs. M and T vs. R, respectively). “M” and “R” samples were similar in richness. Shannon diversity showed the same trend, as “T” was the section having lowest diversity (p=0.012 and p=0.003, T vs. M and T vs. R, respectively) while “M”, “R” did not differ (p=0.43). For inverse Simpson diversity, the trend between root sections is maintained, except that “T” is no longer different “M” but still significantly lower than “R” (p=0.009, T vs. R). Subscale sample types “T” and “M” were significantly lower in Shannon and inverse Simpson diversity than zero (the mean of standardized homogenized root “H”) (Fig. 2A).
Fig. 2. Alpha diversity (A) and beta diversity (B) of the rhizosphere microbiota at different root sections. (A) Alpha diversity (observed richness, Shannon and inverse Simpson diversity) of sections “T”, “M” and “R” was standardized by “H” samples. Each dot refers to a rhizosphere sample. The black dots refer to the mean of standardized alpha diversity. Vertical black lines are the standard deviation. Horizontal black lines are the value of zero. Significance levels shown in the upper panel are from two-sided and two-sample t-test; Significance levels shown in the lower panel are from two-sided and one-sample t-test with a reference value of zero. * indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001. (B) Distribution of different sample types based on weighted UniFrac visualized with principle coordinates analysis (PCoA) with ellipse encircling 75% of samples per condition.

To investigate the overall structure of the rhizosphere microbiota in different root sections, weighted and unweighted UniFrac distance were assessed and visualized with principle coordinates analysis (PCoA) (Fig. 2B, Fig. S2). Although groups were partially overlapped, they all differed from each other in microbial composition (Supplementary Table 1). Traditional “H” samples were less dispersed than any other subscale samples based on weighted UniFrac distance (Supplementary Table 2). In contrast, “T” samples were the most dispersed in terms of unweighted UniFrac distance (Fig. S2, Supplementary Table 2).
To decipher taxonomic differences of root microbial composition among different sample types ("T", "M", "R" and "H"), we depicted the microbial composition at phylum level with a bar-plot (Fig. 3A). The most dominant phylum was Proteobacteria with a relative abundance of 41.9%, followed by Chloroflexi (14.0%), Firmicutes (13.1%), Patescibacteria (7.6%) and Bacteroidetes (6.7%), with these five phyla representing 83.4% of sequences. Of interest, "T" (23.6%) had much more Firmicutes than "M" (10.2%) and especially "R" (5.4%). While Patescibacteria and Verrucomicrobia were more abundant in
“R” (13.1% and 6.7%) than “T” (3.5% and 2.3%) and “M” (6.3% and 3.0%). “M” was at an intermediate level between “T” and “R”. Next, we identified taxa that differed quantitatively amongst three sections at all taxonomic levels (Supplementary Table 3) and discriminant taxa so-obtained at ASV level were shown (Fig. 3B, Fig. S3). Respectively, we found that 9 phyla, 14 classes, 24 orders, 32 families and 39 genera were differentially enriched along the root axis among different root sections. Consistent with what we visually observed from the phylum bar-plot (Fig. 3A), many ASVs from phylum Firmicutes were highly enriched in “T” but depleted or absent in “R”. A number of ASVs from Patescibacteria and Verrucomicrobia were only enriched in “R”.

3.2 Within and between plant variability

To compare to which extent microbiota varied among multiple root samples from the same plant (within plant variability) and among root section from different plants (between plant variability), we assessed the pairwise weighted UniFrac distance of all possible comparisons (Fig. 4). Based weighted UniFrac distance, root section (not including “H” samples) explained 13% of variance (p<0.001) compared to 27% of variance (p<0.001) explained by plant identity. For unweighted UniFrac distance, 5.7% (p<0.001) and 22.9% (p<0.001) of variance were explained by root sections and plant identity, respectively. In detail, we observed that within plant root tips “T” had the largest median pairwise distance (0.26) than middle root “M” (0.23) and rear root “R” (0.19) (p=0.002, T~T vs. M~M; p<0.001, T~T vs. R~R). The distance among “T” samples was same as the distance between “T” and “M” samples (p=0.61, T~T vs. T~M), but significantly smaller than the distance between “T” and “R” (p<0.001, T~T vs. T-R). In addition, between plants, the root sections did not maintain the decreasing trend of within plant variability from “T” and “M” to “R” (p=0.30, T~T vs. R~R, between plants). Comparison between homogenized root “H” yielded the most similar profiles (0.23, between plant) compared to other between plants comparisons.
Fig. 4. Pairwise weighted UniFrac distance between samples from within plant and between plants. Numbers shown are the median value of weighted UniFrac distance under corresponding comparisons. For interpretation sake, Unifrac varies from 0 to 1 and quantifies how dissimilar microbiota are from a phylogenetic basis (0 = identical, 1 = completely different).

We also compared the unweighted UniFrac distance within and between plants (Fig. S4). Two distance metrics showed a similar trend, but a new tendency was displayed in unweighted UniFrac distance. For instance, within T~T (0.51) was no longer different from T~R (0.51) (p=0.57). A stronger individual variation effect was observed (the smallest within plant distance [0.52] was larger than the largest between plant distance [0.51]). “H” samples still showed the highest similarity (0.47, between plant) compared to any other within plant comparisons.

3.3 Impact of inoculants

We then wanted to investigate how root microbiota developed in different sections when spiked with an inoculum compared to control plants colonized from the environmental setting. First, we compared the standardized alpha diversity (observed richness, Shannon and inverse Simpson diversity) of each root section between inoculated and control plants (Fig. 5A). The microbiota in “R” samples represented higher richness (p<0.001) and Shannon diversity (p=0.017) but not for inverse Simpson index in inoculated group than non-inoculated group, indicating the driving effect of OTU richness. However, inverse Simpson revealed a decreased evenness in the “T” section due to inoculation. We also
performed one-sample t-test with a reference value of zero (the mean of standardized “H” samples). “R” samples showed even higher richness than “H” samples (p<0.001).

Fig. 5. Standardized alpha diversity of root sections (A) and weighted UniFrac distance between sections and inoculants (B). The alpha diversity and UniFrac distances of root sections were standardized by “H” samples. (A) Comparisons of standardized alpha diversity (observed richness, Shannon and inverse Simpson diversity) between non-inoculated and inoculated groups. (B) Comparisons of standardized UniFrac distance between root sections and inoculants. Each dot refers to a sample. The mean and standard deviation of standardized alpha diversity and UniFrac distance are shown with black dots and vertical black lines, respectively. Horizontal black lines are the value of zero. Significance levels shown in the upper panel are from two-sided and two-sample t-test; Significance levels shown in the lower panel are from two-sided and one-sample t-test with a reference value of zero. * indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001.

To further confirm the distinct influence of inoculants on root sections, we compared the weighted UniFrac distance between samples from different root sections and the inoculants from corresponding generations (Fig. 5B). In line with alpha diversity, the mean standardized distance of T~Inoculant did not differ between two groups, but M~Inoculants (p=0.007) and R~Inoculants (p<0.001) differed significantly between groups, indicating higher similarity with the inoculant in these sections. Furthermore, “M” and “R” samples in inoculated group showed smaller distances to inoculants than “H” (p<0.001 and p<0.001 for M~Inoculants and R~Inoculant compared to the reference value of zero).
To further address how the taxonomic composition in each root sections was influenced by inoculants, we applied a linear model to discover the ASVs that differed in abundance between groups for each root section (Fig. 6A). We found 144 significant ASVs in section “R” between groups, however, only 24 and 2 ASVs were observed in “M” and “T”, respectively (Fig. 6B). Such divergence in the number of significant taxa across root sections remained at higher taxonomic levels (data not shown). Only 9 ASVs were influenced by inoculants for “H” samples between groups.

Fig. 6. ASVs that differed quantitatively between non-inoculated and inoculated groups. (A) The relative abundance (log10 transformed for visualization) of significant ASVs between groups. Dots are the ASVs. Same ASVs between groups are linked by lines. Dots and lines are colored by the phylum where ASVs are from. (B) Bar-plot showing the number of significant ASVs at each root section and colored by their respective phylum. An ASV is grouped into “Increase” if its abundance is higher in inoculated group than non-inoculated group, otherwise grouped into “Decrease”.

4. Discussion
In the present study, we investigated the rhizosphere microbiota of *Brachypodium distachyon*, a model plant of cereal species, in multiple ways with regards to sampling scale (traditional vs. subscale), location on the root (root sections), within and between plant variations, and effect of inoculations.
Richness and evenness were lowest at root tip (“T”), but climbed quickly and represented similar values for middle (“M”) and rear root (“R”) (Fig. 2A). It concurs with Kawasaki’s work (Kawasaki et al., 2016), showing that the root base had higher Shannon diversity than root tip. Besides, the stability of rhizosphere microbial communities increased from tip root to rear root (Fig. 4). This pattern was associated with clear taxonomic signatures indicating that root tip samples were more dispersed than root base (Fig. S2, Supplementary Table 2). We speculate that the high variability of root tip was due to several reasons. First, different root sections have different ages. Root tip (“T”) is a section of young age and highly active in the secretion of root exudates (Dennis et al., 2010). Therefore, microbes residing in the rhizosphere of root tip are likely r-strategists, fast in growth, reproduction, and response to carbon resources (Fig. 3A) resulting in a dynamic and heterogeneous microbial structure. In contrast, “M” and especially “R” are older root parts having limited rhizodeposits compared to “T” and are preferred by k-strategists which often form stable microbial communities. Second, the priority effect might play a more important role in the root tip than root base. As plant roots grow through soil, random microorganisms in their pathway will be the first colonizers. Therefore, the order and timing of arrival matter, as it will enrich microbial communities differently at root tip (Fukami et al., 2007). But microbial communities tend to stabilize over time as the plant age resulting in more stable communities in “M” and “R”. Such priority effect regarding fungi in root tip has been reported (Kennedy and Bruns, 2005; Morris et al., 2008; Kennedy et al., 2009), and we show here it applies to bacteria as well.

Consistent with our speculation, we found root tip was significantly more enriched with Firmicutes compared to middle and rear sections (“T” [23.7%], “M” [10.2%] and “R” [5.4%]; Fig. 3A). In line with the work by Cleveland, Firmicutes (primarily Bacillus at genus level) were shown to respond to the addition of labile C compounds quickly (Cleveland et al., 2007). The signature of enriched Firmicutes (Bacillus at the genus level, Supplementary Table 3) in root tips might reflect the large amount of carbon released from a fast-growing root tip. In contrast, rear root showed a significantly higher amount of Verrucomicrobia which is a phylum behaving as k-strategists (Aguirre-von-Wobeser et al., 2018).

We found that plant identity (individual variation) was a strong influencing factor and can shape the heterogeneity of root microbiota in different root sections. Plant identity accounted for a higher proportion of the variance than root sections (27% vs. 13%, weighted UniFrac; 22.9% vs. 5.7%, unweighted UniFrac). Larger within plant UniFrac
distances were observed than between plant (Fig. 4). Besides, the decreasing variability from tip to base within an individual was shifted when compared between individuals. It suggests that the individual variation occurring between sections in different replicate plants (coming from the same seed batch and grown in the same controlled environmental conditions) is higher than variation found within the same plant amongst its roots. But the traditional sampling approach is benefited from their large scale where these small variations were averaged and diminished (Fig. 4).

Interestingly, we found that from tip to base, rhizosphere microbiota was increasingly affected by our inoculants (Fig. 5), by comparing the alpha diversity, weighted UniFrac distance, and taxa abundance of each root section to the controls. It is obvious for the rear section in inoculated group that 14 ASVs were newcomers introduced via inoculants since they were absent in non-inoculated group (Fig. 6). Such evidence indicates that root tip was relatively resistant to inoculants and the colonization capacity of the inoculants was mainly located at the root base. We speculated that it was because root tips were continuously encountering new microbes while growing through soil. After 4 weeks of growth, this has resulted in the progressive displacement of the initially colonized microbes introduced by inoculation, which were replaced by ambient microbes. Nevertheless, still few microbes might colonize root tip persistently and reproduce rapidly which disturbed the evenness of local microbial communities and led to a lower evenness (inverse Simpson diversity) (Fig. 5). Such as the very abundant ASV from class Alphaproteobacteria (identified as Xanthobacteraceae at family level) was consistently enriched in inoculated group at all sample types including root tip (Fig. 6A). In contrast, the root base is a relatively static environment that can offer the opportunity for the inoculated microbes to colonize, reproduce and outcompete less fit soil bacteria. However, these observations could be confounded by the inoculation method since the inoculants were applied on top of the superficial soil, leading to more microbes establishing on the top than migrating downwards, therefore resulting in a decreasing inoculation influence towards subsoil.

In terms of taxonomic composition, traditional samples resembled the mixture of different root sections having a high amount of Firmicutes (the feature of “T”), and a similar amount of Patescibacteria and Verrucomicrobia compared to “M” and “R”, respectively. Hence, rhizosphere microbial communities in “H” samples were most diverse and evenly distributed (Fig. 2A), least dispersed (Fig. 2B and Supplementary Table 2), and represented high pairwise similarity (Fig. 4B), compared to smaller scale sample types.
However, traditional samples were naturally limited to assess the influence of individual variation and inoculation on rhizosphere microbiota. For instance, at subscale, our results showed that plant identity (individual variation) explained up to 27% of variance in beta diversity, but not able to assess such effect at a larger scale. “H” samples were expected to contain more information about inoculation, while, only 9 ASVs were influenced by inoculation (Fig. 6). It might be the consequence of traditional sampling approach that the entire root system is homogenized (Barillot et al., 2013). In contrast, the subscale “R” section was shown to be the hotspot for strong interaction between plant roots and inoculum.

In conclusion, our results showed that rhizosphere is heterogeneous along root axis. Root tip had lower diversity, higher variation in taxonomic composition and being less influenced by inoculation, compared to the middle section and root base. Firmicutes was more enriched at root tip and Patescibacteria and Verrucomicrobia were more represented at root base. Hence, the root microbiome seemed to be strongly influenced by microbial interactions at microscale such as strong competition and stochastic colonization at the root tip, but more studies are needed to identify the drivers for microscale variability in microbiome structure. However, when homogenizing the entire root system, these important features of rhizosphere microbiota will be overlooked.

**Acknowledgments**

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**Reference**


Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBNet.journal 17: 10


Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH (2013) Going back to the roots: the


Supplementary material

Fig. S1. Rarefaction curves for observed richness, Shannon and inverse Simpson diversity. The observed richness almost reached saturation before 10,000 reads, whereas Shannon and inverse Simpson diversity were close to maximum after 1000 reads. The black vertical lines show the sequencing depth of 2000 reads. Dots are the mean of alpha diversity in corresponding groups and linked by lines. Colored vertical bars are the standard deviation of alpha diversity obtained from 100 random rarefactions at corresponding sequencing depth. “extr-ctrl” refers to extraction negative controls; “PCR-ctrl” refers to PCR negative controls. These negative controls were neither inoculated nor non-inoculated but mirrored in figure panels as a reference.
Fig. S2. Unweighted UniFrac distances of different root sections visualized with principal coordinates analysis (PCoA), with ellipses encircling 75% of samples from each group.

Fig. S3. Bar-plot showing the number of significantly enriched ASVs (relative abundance larger than 0.01%) in root sections. ASVs are colored by their respective phylum. An ASV is assigned to a root section if it is more enriched in this section than others.
Fig. S4. Pairwise unweighted UniFrac distance between samples from within plant and between plants. Numbers shown are the median value of weighted UniFrac distance under corresponding comparisons. For interpretation sake, Unifrac varies from 0 to 1 and quantifies how dissimilar microbiota are from a phylogenetic basis (0 = identical, 1 = completely different).

Supplementary Table 1
The pairwise comparison of beta diversity (weighted and unweighted UniFrac distance) between sample types (PERMANOVA, 10,000 permutations)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>p values (weighted UniFrac)</th>
<th>p values (unweighted UniFrac)</th>
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</thead>
<tbody>
<tr>
<td>T~M</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>T~R</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>T~H</td>
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<td>&lt; 0.001</td>
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<tr>
<td>M~R</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>M~H</td>
<td>&lt; 0.001</td>
<td>0.127</td>
</tr>
<tr>
<td>R~H</td>
<td>&lt; 0.001</td>
<td>0.062</td>
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Supplementary Table 2
The pairwise comparisons of multivariate homogeneity of group dispersions (variances).

<table>
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<th>Comparison</th>
<th>p values (weighted UniFrac)</th>
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<td>T~M</td>
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<td>T~R</td>
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<td>T~H</td>
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<td>M~R</td>
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<td>R~H</td>
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### Supplementary Table 3

The relative abundance of discriminant taxa between root sections at different phylogenetic levels.

<table>
<thead>
<tr>
<th>Taxonomic classification</th>
<th>Sample types (mean ± SD)</th>
<th>p values adjusted (FDR&lt;0.05)</th>
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<td><strong>Phylum</strong></td>
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<td>Firmicutes</td>
<td>23.60% ± 17.07,a</td>
<td>10.18% ± 6.41,b</td>
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<td>Patescibacteria</td>
<td>3.49% ± 3.03,b</td>
<td>6.30% ± 5.48,b</td>
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<td>Verrucomicrobia</td>
<td>2.27% ± 1.79,b</td>
<td>2.99% ± 1.86,b</td>
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<td>Bacteroidetes</td>
<td>4.73% ± 2.47,b</td>
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<td>Gemmatimonadetes</td>
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<td>Deinococcus_Thermus</td>
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<td>Armatimonadetes</td>
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<td>Bacillales</td>
<td>20.77% ± 16.92,a</td>
<td>9.67% ± 6.22,b</td>
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<td>0.22% ± 1.13,b</td>
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<td>1.01% ± 1.54,a</td>
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<td>2.18% ± 2.05, c</td>
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**Family**

- **Alicyclobacillaceae**: 6.57% ± 14.17, a 1.40% ± 4.71, b 0.16% ± 0.19, b 0.18% ± 0.19 7.93E-03
- **Bacillaceae**: 11.53% ± 8.36, a 6.79% ± 3.85, b 4.62% ± 2.32, b 17.96% ± 4.26 6.57E-07
- **Veillonellaceae**: 1.64% ± 4.57, a 0.22% ± 1.13, b 0.02% ± 0.05, b 0.00% ± 0.00 4.21E-02
- **Pseudomonadaceae**: 1.96% ± 3.64, a 1.16% ± 1.13, a 0.43% ± 0.30, b 1.88% ± 0.93 2.69E-02
- **Rubritaleaceae**: 0.46% ± 1.14, b 0.64% ± 1.25, b 2.80% ± 4.19, a 3.28% ± 4.35 4.83E-04
- **A4b**: 1.46% ± 1.46, c 2.67% ± 2.14, b 4.20% ± 2.63, a 1.21% ± 1.52 2.70E-06
- **Deinococccaeae**: 0.53% ± 1.25, b 0.55% ± 1.15, b 1.60% ± 2.33, a 0.65% ± 0.78 1.06E-02
- **Gemmatimonadaceae**: 2.39% ± 1.80, ab 3.10% ± 2.00, a 1.69% ± 1.13, b 0.51% ± 0.35 1.33E-03
- **Fimbrimonadaceae**: 0.39% ± 0.35, b 0.48% ± 0.58, b 1.06% ± 1.70, a 1.65% ± 1.67 1.60E-02
- **Fibrobacteraceae**: 0.20% ± 0.39, b 0.26% ± 0.84, b 1.48% ± 1.93, a 0.06% ± 0.10 5.12E-06
- **Chthoniobacteraceae**: 0.85% ± 0.73, b 1.21% ± 1.23, b 1.80% ± 1.26, a 2.62% ± 1.65 1.74E-03
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<td>Verrucomicrobiaceae</td>
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<td>Microbacteriaeae</td>
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<td>Rhizobacter</td>
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<td>Deinococcus</td>
<td>0.50% ± 1.24,b</td>
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<td>Chthoniobacter</td>
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<td>Bradyrhizobium</td>
<td>0.68% ± 0.98,a</td>
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<tr>
<td></td>
<td></td>
<td>Devosia</td>
<td>1.09% ± 0.97,b</td>
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</table>

**Genus**

- Devosia: 1.09% ± 0.97, b
- Bradyrhizobium: 0.68% ± 0.98, a
- Deinococcus: 0.50% ± 1.24, b
- Chthoniobacter: 0.85% ± 0.73, b
- Paenibacillus: 0.81% ± 1.14, a
- Sorangium: 0.10% ± 0.30, b
- Bradyrhizobium: 0.68% ± 0.98, a
- Devosia: 1.09% ± 0.97, b
- Peptostreptococcaceae: 0.40% ± 0.76, a
- Verrucomicrobiaceae: 0.31% ± 0.54, b
- Pedosphaeraceae: 0.38% ± 0.33, b
- Hymenobacteraceae: 0.44% ± 0.36, b
- Streptococcaceae: 0.23% ± 0.63, a
- Planococcaceae: 0.66% ± 0.63, a
- Intrasporangiaceae: 0.19% ± 0.43, a
- Sphingobacteriales_KD3_93: 0.03% ± 0.09, b
- Rhodanobacteraceae: 0.16% ± 0.21, b
- WD2101_soil_group: 0.15% ± 0.15, b
- AKYG1722: 0.07% ± 0.14, b
- Propionibacteriaeae: 0.07% ± 0.14, a
- Microbacteriaeae: 0.00% ± 0.00, b
- Myxococcales_mle1_27: 0.02% ± 0.04, b

**Notes:**
- Values are given as mean percentage ± standard deviation.
- Different letters (a, b) indicate significant differences.
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<thead>
<tr>
<th>Species</th>
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<th>Mean ± SD</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
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<td>Oligoflexus</td>
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<td>0.57% ± 0.72,a</td>
<td>0.16% ± 0.11,b</td>
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<td>0.97% ± 0.80,a</td>
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<td>Sporacetigenium</td>
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<td>0.09% ± 0.12,b</td>
<td>0.02% ± 0.04,b</td>
<td>0.07% ± 0.09</td>
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<td>Altererythro bacter</td>
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<td>0.13% ± 0.10,b</td>
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<td>0.12% ± 0.10,b</td>
<td>0.03% ± 0.04</td>
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<td>Enticicia</td>
<td>0.04% ± 0.17,b</td>
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<td>0.05% ± 0.06</td>
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<td>0.06% ± 0.13,a</td>
<td>0.03% ± 0.05</td>
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<td>0.03% ± 0.06,b</td>
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p values were from the three-level (T, M and R) two-way ANOVA and the time effect (generation) was adjusted. "H" samples were included as a reference. Alphabet letters after mean ± SD are the Tukey’s post-hoc test and different letters represent different levels of significance. The false discovery rate in multiple comparisons was controlled with Benjamini–Hochberg method.
Co-authorship Statements
### 1. PhD student

<table>
<thead>
<tr>
<th>Name</th>
<th>Shaodong Wei</th>
<th>UCPH user id:</th>
<th>qvw161</th>
</tr>
</thead>
<tbody>
<tr>
<td>Department</td>
<td>Biology</td>
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### 2. Paper/Manuscript

**This co-authorship declaration applies to the following:**

<table>
<thead>
<tr>
<th>Title</th>
<th>Short- and long-term impacts of azithromycin treatment on the gut microbiota in children: A double-blind, randomized, placebo-controlled trial</th>
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<tbody>
<tr>
<td>Authors(s)</td>
<td>Shaodong Wei, Martin Steen Mortensen, Jakob Stokholm, Asker Daniel Brejnrod, Jonathan Thorsen, Morten Arendt Rasmussen, Urvish Trivedi, Hans Bisgaard, Søren Johannes Sørensen</td>
</tr>
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<td>Vol/page</td>
<td>38/265-272</td>
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</table>

### 3. Contributions to the paper/manuscript made by the PhD student

**What was the role of the PhD student in designing the study?**

None.

**How did the PhD student participate in data collection and/or development of theory?**

The PhD student did the DNA extraction, sequencing, data analysis and data interpretation.

**Which part of the manuscript did the PhD student write or contribute to?**

The entire manuscript was written by the PhD student, then revised by co-authors.

**Did the PhD student read and comment on the final manuscript?**

Yes.
4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master's degree)?

☐ Yes

Please indicate which degree/thesis:

Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

---

Signatures

The co-author statement should always be signed by the first author, the corresponding-/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

Date: 28/02/2019 Name: Søren Johannes Sørensen Signature: [Signature]

Date: 28/02/2019 Name: Shaodong Wei Signature: [Signature]

Date: Name: Signature: [Signature]

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but not included in the PhD thesis. You can find your PhD secretary here: [www.science.ku.dk/phd/](http://www.science.ku.dk/phd/)
3. Contributions to the paper/manuscript made by the PhD student

What was the role of the PhD student in designing the study?

None.

How did the PhD student participate in data collection and/or development of theory?

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Did the PhD student read and comment on the final manuscript?

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4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master’s degree)? □ Yes

Please indicate which degree/thesis:

Please indicate which specific part(-s) of the paper that has been produced as part of the PhD study:

Signatures

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

Date: 28/02/2019  Name: Søren Johannes Sørensen  Signature: 

Date: 28/02/2019  Name: Shaodong Wei  Signature: 

Date:  Name:  Signature: 

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but not included in the PhD thesis. You can find your PhD secretary here: www.science.ku.dk/phd/
3A. Co-authorship statement
All papers/manuscripts with multiple authors which is part of a PhD thesis should contain a co-author statement, stating the PhD student's contribution to the paper.

<table>
<thead>
<tr>
<th>1. PhD student</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name:</strong> Shaodong Wei</td>
</tr>
<tr>
<td><strong>Department:</strong> Biology</td>
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<table>
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<tr>
<td><strong>Title:</strong> A glance into spatial and individual variability of rhizosphere microbiota</td>
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<tr>
<td><strong>Authors(s):</strong> Shaodong Wei, Samuel Jacquiod, Manuel Blouin, Laurent Philippot, Søren Johannes Sørensen</td>
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<td><strong>Journal:</strong></td>
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<thead>
<tr>
<th>3. Contributions to the paper/manuscript made by the PhD student</th>
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<td><strong>What was the role of the PhD student in designing the study?</strong></td>
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<td>The PhD student came up with ideas.</td>
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<td><strong>How did the PhD student participate in data collection and/or development of theory?</strong></td>
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<tr>
<td>The PhD student participated in the study design and did sample collection, DNA extraction, sequencing, data analysis and data interpretation.</td>
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<td><strong>Which part of the manuscript did the PhD student write or contribute to?</strong></td>
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<td>The entire manuscript was written by the PhD student, then revised by co-authors.</td>
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<td><strong>Did the PhD student read and comment on the final manuscript?</strong></td>
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<td>Yes.</td>
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4. Material in the paper from another degree / thesis

Data collected and preliminary work carried out as part of another degree/thesis may be part of the PhD thesis if further research, analysis and writing are carried out as part of the PhD study.

Does the paper contain data material, which has also formed part of a previous degree / thesis (e.g. your master's degree)? □ Yes

Please indicate which degree/thesis:

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Signatures

The co-author statement should always be signed by the first author, the corresponding/senior author and the PhD student. If there are two or three authors the statement must always be signed by them all.

Date: 28/02/2019  Name: Søren Johannes Sørensen  Signature:

Date: 28/02/2019  Name: Shaodong Wei  Signature:

Date:  Name:  Signature:

The co-authorship statement must be handed in to the PhD secretary at your department at the same time as the PhD thesis, but not included in the PhD thesis. You can find your PhD secretary here: www.science.ku.dk/phd/