Ecotoxicology and Environmental Safety 142 (2017) 164–170

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Comparing the impact of ultrafine particles from petrodiesel and biodiesel combustion to bacterial metabolism by targeted HPLC-MS/MS metabolic profiling

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ARTICLE INFO

Keywords:
Ultrafine particles
Targeted metabolic profiling
Bacteria metabolism
Diesel combustion
Multivariate statistical analysis

ABSTRACT

Alterations of gut bacterial metabolism play an important role in their host metabolism, and can result in diseases such as obesity and diabetes. While many factors were discovered influencing the gut bacterial metabolism, exposure to ultrafine particles (UFPs) from engine combustions were recently proposed to be a potential risk factor for the perturbation of gut bacterial metabolism, and consequentially to obesity and diabetes development. This study focused on evaluation of how UFPs from diesel engine combustions impact gut bacterial metabolism. We hypothesize that UFPs from different type of diesel (petrodiesel vs. biodiesel) will both impact bacterial metabolism, and the degree of impact is also diesel type-dependent. Targeted metabolic profiling of 221 metabolites were applied to three model gut bacteria *in vitro*, *Streptococcus salivarius*, *Lactobacillus acidophilus* and *Lactobacillus fermentum*. UFPs from two types of fuels, petrodiesel (B0) and a biodiesel blend (B20: 20% soy biodiesel/80% B0 by volume), were exposed to the bacteria and their metabolic changes were compared. For each bacterial strain, metabolites with significantly changed abundance were observed in both perturbations, and all three strains have increased number of altered metabolites detected from B20 UFPs perturbation in comparison to B0 UFPs. Multivariate statistical analysis further confirmed that the metabolic profiles were clearly different between testing groups. Metabolic pathway analyses also demonstrated several important metabolic pathways, including pathways involves amino acids biosynthesis and sugar metabolism, were significantly impacted by UFPs exposure.

1. Background

The health impact of air pollution has been studied extensively in recent years (Fukagawa et al., 2013; Künzi et al., 2015; Stanek et al., 2011). Exposure to air pollutants such as particulate matter (PM), especially fine PM and ultrafine particles (UFPs, particulate matter of nanoscale size, generally less than 100 nm in diameter), has been linked to pulmonary and cardiovascular diseases, as well as to the increased hospital admissions and mortality (Künzi et al., 2015; Nel, 2005; Stanek et al., 2011). Air pollution PM is also associated with obesity and related diseases; however, the mechanistic underpinnings of these adverse metabolic effects are unclear (Balti et al., 2014; Paglia et al., 2016; Sun et al., 2009; Xu et al., 2010). Research in the last decade also suggests that human gut microbiota (which is primarily bacteria) can play an important role in the regulation of human health and diseases. Published literature suggests that gut microbial metabolic activities can affect host energy storage by influencing nutrient metabolism (Heinken and Thiele, 2015; Musso et al., 2011). Gut microbiota are considered important factors in obesity and diabetes, yet little is known about how they interact with environmental factors (especially with air pollution PM), and the consequences to the disease phenotype. It is known that the gastrointestinal tract is exposed to high concentrations of pollutant PM, either by mucociliary transport of inhaled PM from the lungs into the intestine, or by PM-contaminated food and water (Beamish et al., 2011; Salim et al., 2014). However, the molecular events revealing how environmental factors can provoke the gut bacteria metabolic consequences are still poorly understood.

On the other hand, the generally greener and environmental friendly biodiesel usage in the United States have been promoted over the past decade, even though the current usage of biodiesel is still small compare to the usage of petrodiesel, there is an increased trend for the use of biodiesel as alternative fuel for transportation as well as other

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http://dx.doi.org/10.1016/j.ecoenv.2017.04.002
Received 11 November 2016; Received in revised form 29 March 2017; Accepted 3 April 2017
Available online 13 April 2017

industries (HEI, 2013). Hence, there are critical needs for us to understand the health and environmental effects of biodiesel combustion due to the expected increase use of biodiesel in the future, and so far only limited data is available for the health effects of biodiesel emissions (Fukagawa et al., 2013; Gerlofs-Nijland et al., 2013; Hemmingsen et al., 2011).

Therefore, in this proof-of-concept study, we aimed to answer two basic research questions, whether the UFPs generated by diesel fuel combustion will impact gut bacterial metabolism, and whether the impact is even more/less severe due to different types of diesel fuel used for the UFP generation. Three model bacteria, *Streptococcus salivarius* (*S. salivarius*), *Lactobacillus acidophilus* (*L. acidophilus*) and *Lactobacillus fermentum* (*L. fermentum*) were tested *in vitro* in this study. These representative bacteria were frequently detected and measured in human gut microbial flora and have been connected to different health effect to the host, such as degradation of bile salt, modulation of intestinal pain and cholesterol reduction (Drasar and Shiner, 1969; Hill and Drasar, 1968; Pereira and Gibson, 2002; Rousseaux et al., 2007). Each bacterium was exposed to either petrodiesel combustion generated UFPs (B0-UFPs) or biodiesel combustion generated UFPs (B20-UFPs). A targeted metabolic profiling approach is applied to systematically investigate over two hundred metabolites, which by definition is the measurement of defined metabolites groups that were chemically characterized and annotated. Using internal standards, targeted metabolic profiling analysis can be undertaken in a semi-quantitative manner. The metabolite targets were selected from several previous studies to cover important metabolic pathways (Bajad et al., 2006; Yuan et al., 2012; Zhu et al., 2015, 2014), and their measurement reproducibility have been carefully evaluated and optimized (Schelli et al., 2016; Xu et al., 2017). The detected and identified metabolites are ready for further interpretation within their biological context. Using this targeted metabolic profiling approach, we discovered that the metabolic activities were vastly disturbed by UFPs exposure, as indicated by number of metabolites with significant changes in UFPs exposure tests, and by the metabolic pathways impact analyses. Furthermore, the number of metabolites that with significant changes were generally higher in biodiesel exposure test compare to the petrodiesel exposure test, revealed interesting fact that the bacterial level metabolic perturbation from biodiesel UFPs may be even severe than the traditional petrodiesel. To our best knowledge, this is the very first study to specifically look at the impact of UFPs exposure to bacterial metabolism using targeted mass spectrometry based metabolomics approach. While we acknowledge that we are only demonstrating our findings with limited number of gut bacteria, and the bacteria cultivation system is simplified compared to the real human gut environment. We expect our discovery work can initiate the discussion of the role of gut bacteria in the environmental health studies, can lead to better understanding of how environmental factors interfere with human gut bacteria and their metabolic activities, and can provide fundamental information towards *in vivo* studies of their influence to host metabolism in the future.

2. Methods

2.1. Ultrafine particle generation, collection and characterization

UFPs were generated, collected and characterized from the University of Vermont Transportation Air Quality laboratory as described previously (Fukagawa et al., 2013). Briefly, a 4-cylinder, 1.9 l Volkswagen light-duty diesel engine and Klam dynamometer (Armfield CM-12) were operated at various throttle and brake settings to enable triplicate time-resolved measurement of particle number distributions with an engine exhaust particle sizer (EEPS, TSI, Inc.). Particles for chemistry and gravimetric mass were collected on 47 mm side-by-side Teflon-coated glass fiber and Teflon filters, and side-by-side 60 mL Teflon impingers. Filter samples were collected from raw exhaust without dilution, but EEPS particle distribution were measured after single-stage dilution with HEPA-filtered, dry (silica gel) room air at a dilution ratio of ~80. After post weighing, filter samples were stored at −80 °C. Blank runs were performed identically without starting the diesel engine for 50 min. Filter and impinger samples were collected over 75 min periods that do not include engine start and warm-up (defined by stable coolant temperature, 92 °C). Two fuel compositions, ultralow sulfur petrodiesel and certified soy-based biodiesel blended at 20% by volume, were used. The B20 will be blended from the same petrodiesel and confirmed by FTIR (IROX-D).

2.2. Preparation of particle stock suspensions

Raw exhaust particles collected in ethanol impingers were concentrated via gentle N2 blowdown to generate stock suspensions of approximately 1 mg/mL. PM concentrations of impinged suspensions were determined in triplicate using gravimetric analysis (Cahn C-31 µg balance, 0.001 mg sensitivity) of 100 µL aliquots prepared prior to, during, and after dilution of the stock suspension with sterilized Milli-Q water (by autoclave) to obtain aqueous solutions (Fukagawa et al., 2013).

2.3. Bacterial strains, growth condition and ultrafine particle exposure

Three model bacteria, *Streptococcus salivarius* (*S. salivarius*) ATCC 13419, *Lactobacillus acidophilus* (*L. acidophilus*) ATCC 4356 and *Lactobacillus fermentum* (L. *fermentum*) ATCC 9338 were purchased from American Type Culture Center (ATCC) and used in this study. Fresh bacterial culture was prepared from freezer stock by standard microbiology operation, and grown in either brain heart infusion broth or *Lactobacilli* MRS Broth at 37 °C. *L. acidophilus* and *L. fermentum* were incubated in fully anaerobic condition within an anaerobic chamber (COY Lab Products, MI, USA). The bacteria were firstly cultured for 16–48 h (depending on their growth rates), to reach sufficient number of bacterial cells (generally ~10^9 colony forming unit (CFU)/mL), then half of the culture replicates (n = 6) were mixed with UFPs to reach final particle concentration of 20 µg/mL, with the other half served as control group (n = 6). The exposure dose was determined by our preliminary test, and is comparable to previously reported study (Fukagawa et al., 2013). Cultures with or without UFPs exposure were incubated for another 6 h before the bacterial cells were harvested for metabolites extraction. Optical density (OD_{600}) of each bacterial culture was also recorded for each step during the experiments.

2.4. Metabolite extraction and sample preparation

After the exposure experiments, bacterial culture was centrifuged at 14,000 rpm for 10 min, then the supernatant was removed, the cell pellets were washed three times by phosphate buffer saline (PBS) and filtered by 0.22 µm pore size filter to get rid of the UFPs. Afterwards, cold methanol was added to bacterial cell pellets for metabolite extraction, together with a mixture of 20 stable isotope labeled amino acids (U-13C and U-15N, purity 97–99%, Cambridge Isotope Laboratory, MA, USA) as internal standards to ensure the reproducibility and robustness of the method. Extracted methanol solution was dried under vacuum and reconstituted using mixture of HPLC grade water and acetonitrile (Fisher Scientific, PA, USA) and then loaded into LC vials for HPLC-MS/MS analysis. Targeted metabolic profiling of 221 metabolites was utilized to confidently detect the altered metabolic activities caused by UFPs exposure. Quality control (QC) samples (consist of pooled bacterial metabolites from randomly chosen samples in actual sample set) were run in between every ten samples to ensure proper instrument performance, and to provide basis for necessary data normalization. Blanks (mixture of HPLC grade methanol and water) were also run intermediately to ensure there is no carry over from sample to sample.