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- Dietary emulsifiers alter composition and activity of the human gut
   microbiota *in vitro*, irrespective of chemical or natural emulsifier origin.
- 3 Lisa Miclotte<sup>1</sup>, Chris Callewaert<sup>1</sup>, Kim de Paepe<sup>1</sup>, Leen Rymenans<sup>2</sup>, Jeroen Raes<sup>2</sup>, Andreja Rajkovic<sup>3</sup>,
- 4 John Van Camp<sup>4</sup>, Tom Van de Wiele<sup>1\*</sup>
- <sup>5</sup> <sup>1</sup> Center for Microbial Ecology and Technology (CMET), Ghent University, Ghent, Belgium.
- 6 <sup>2</sup> VIB nucleomics core, KULeuven, Ghent, Belgium. Lab for moleculair bacteriology Rega -
- <sup>3</sup> Department of Food technology,- safety and health ,Ghent University, Ghent, Belgium.
- <sup>4</sup> Department of Food technology,- safety and health, Ghent University, Ghent, Belgium.
- 9 \* Correspondence:
- 10 Tom Van de Wiele
- 11 <u>tom.vandewiele@ugent.be</u>
- 12 Keywords: dietary emulsifiers, human gut microbiota, interindividual variablility, microbiome
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# 15 Abstract

16 The use of additives in food products has become an important public health concern. In recent reports,

17 dietary emulsifiers have been shown to affect the gut microbiota, contributing to a pro-inflammatory

18 phenotype and metabolic syndrome. So far, it is not yet known whether similar microbiome shifts are

19 observable for a more diverse set of emulsifier types and to what extent these effects vary with the

20 unique features of an individual's microbiome.

To bridge this gap, we investigated the effect of five dietary emulsifiers on the fecal microbiota from human individuals upon a 48 hour exposure. Community structure was assessed with quantative microbial profiling, functionality was evaluated by measuring fermentation metabolites and proinflammatory properties were assessed with the phylogenetic prediction algorythm PICRUSt, together with a TLR5 reporter cell assay for flagellin. A comparison was made between two mainstream chemical emulsifiers (carboxymethylcellulose and P80), a natural extract (soy lecithin) and biotechnological emulsifiers (sophorolipids and rhamnolipids).

28 While fecal microbiota responded in a donor-dependent manner to the different emulsifiers, profound

29 differences between emulsifier were observed. Rhamnolipids, sophorolipids and soy lecithin

30 eliminated 91%  $\pm$  0%, 89%  $\pm$  1% and 87%  $\pm$  1% of the viable bacterial population after 48 hours, yet

they all selectively increased the proportional abundance of putative pathogens. Moreover, profound shifts in butyrate (-96%  $\pm$  6%, -73%  $\pm$  24% and -34  $\pm$  25% respectively) and propionate (+13%  $\pm$  24

32 since in burylate (-90%  $\pm$  0%, -75%  $\pm$  24% and -54  $\pm$  25% respectively) and propriorate (+15%  $\pm$  24 33 %, +88%  $\pm$  50% and +29%  $\pm$  16% respectively) production were observed for these emulsifiers.

 $33 \quad \%, +38\% \pm 30\%$  and  $+29\% \pm 10\%$  respectively) production were observed for these emulanters. 34 Phylogenetic prediction indicated higher motility, which was, however, not confirmed by increased

35 flagellin levels using the TLR5 reporter cell assay.

36 We conclude that dietary emulsifiers can severely impact the gut microbiota and this seems to be

37 proportional to their emulsifying strength, rather than emulsifier type or origin. As biotechnological 38 emulsifiers were especially more impactful than chemical emulsifiers, caution is warranted when

39 considering them as more natural alternatives for clean label strategies.

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# 40 **1** Introduction

41 The current obesity crisis and related health conditions are increasingly associated with the overconsumption of so-called ultra-processed food products (Waterlander et al. 2018; Broussard and 42 43 Devkota 2016; Branca et al. 2019; Rauber et al. 2018; Carlos Augusto Monteiro et al. 2017). Food 44 additives are characteristic elements of said products (Carocho et al. 2014; C. A. Monteiro et al. 2013; 45 Carlos Augusto Monteiro et al. 2017) and are added to enhance, amongst others, shelf life, palatablility, 46 texture, color and nutritional value. However, the health impact of certain food additives has always 47 been questioned (Miclotte and Van de Wiele 2019; Carocho et al. 2014; Payne, Chassard, and Lacroix 48 2012), and at this moment, the use of food additives in food products is one of the main public concerns 49 about food in Europe (EFSA 2019).

50 Diet is known to have a strong and fast impact on the gut microbiota (Martínez Steele et al. 2017;

50 Diet is known to have a strong and fast impact on the gut incrobiota (Martinez Steele et al. 2017, 51 Musso, Gambino, and Cassader 2010; Ding et al. 2019), which is generally considered an important 52 parameter of gut and overall health (Bischoff 2011; Ding et al. 2019; Musso, Gambino, and Cassader 53 2010). An unbalanced gut microbiota is being related to several physical and mental illnesses and 54 conditions (Ding et al. 2019). With respect to obesity and NCDs, a dysbiosed gut microbiota is 55 characterized by a lower alpha diversity and is related to increased harvest from food and decreased 56 fatty acid oxidation, glucose tolerance, production of satiety hormones and intestinal barrier integrity 57 (Musso, Cambino, and Cassader 2010)

57 (Musso, Gambino, and Cassader 2010).

Recently, research has emerged that ties the consumption of additives to health markers through the gut microbiota. Dietary emulsifiers in particular have been proposed to display a destabilizing impact on gut health. Chassaing et al (2015, 2017) found *in vivo* that sodium carboxymethylcellulose (CMC) and polysorbate 80 (P80) increase gut microbial motility and lower mucus layer thickness, yielding an increased production of pro-inflammatory compounds, low-grade gut inflammation and weight gain.

63 Another study has linked glycerol monolaureate (GML) with signatures of metabolic syndrome 64 together with alterations of gut microbiota composition, among which decreased abundance of

65 Akkermansia muciniphila and increased abundance of Escherichia coli (Jiang et al. 2018). The latter

- study is particularly relevant since GML is one of the World's most widely used dietary emulsifiers(E471).
- 68 Knowledge that is currently still lacking from literature regarding the impact of dietary emulsifiers is
- what characteristics of an emulsifier determine its destabilizing effects, whether alternative, more natural emulsifiers could be safer and to what extent the unique features of an individual's microbiome
- 70 natural emulsiners could be safer and to what extent the unque 71 play a role in the purported effects on the microbiome.

The present study describes the effects of five dietary emulsifiers: CMC, P80, soy lecithin, sophorolipids and rhamnolipids. The first two, CMC and P80 are synthetic emulsifiers that have been used for years and both are considered safe for human oral consumption. CMC is a water soluble anionic polymer with water-binding properties, due to which it is used as a thickener, emulsifier or

76 water-retainer in applications like pharmaceuticals, food products, paper, cosmetics, detergents, etc.

- 77 (Biswal and Singh 2004; Hercules Inc. and Aqualon 1999). In Europe, CMC can be used in many food
- 78 products at *quantum satis* levels <sup>1</sup>(European Commision 2014) and in the USA, CMC caries the GRAS-

<sup>&</sup>lt;sup>1</sup> European Commision. 2014. "00191 - Sodium Carboxymethyl Cellulose - E466." 2014.

https://webgate.ec.europa.eu/foods\_system/main/index.cfm?event=substance.view&identifier=191.

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status (generally recognized as safe) for applications in food <sup>2,3</sup>(FDA 2020, 2019b). P80 is a member of the polysorbates, a group of non-ionic surfactants with applications mainly in the food, cosmetics

81 and pharmaceutical industries (Nielsen et al. 2016; FMI 2020). With an acceptable daily intake of 25

82 mg/kg BW/day (Aguilar et al. 2015), EFSA allows it's use in products like sauces, soups, chewing

- 83 gum, coconut milk, dairy products and usually at maximal concentrations of 10-10000 mg/kg,
- 84 depending on the product <sup>4</sup> (European Commission n.d.). Also the U.S. Food and Drug Administration
- (FDA) limits the use of P80 to 4 10000 mg/kg, depending on the product <sup>5</sup> (FDA 2019a).

Soy lecithin is a mixture of phospoholipids (at least 60%), triglycerides, sterols and carbohydrates 86 obtained by extraction from soybeans. It is more widely used than CMC and P80, primarily in bakery 87 88 products, ice creams, chocolate etc<sup>6</sup> (European Commision 2018). Lecithins are allowed by EFSA in most food applications in quantum satis levels, and also the FDA considers soy lecithin a GRAS 89 compound<sup>7</sup> (Carocho et al. 2014; FDA 2019c). Even though soy lecithin is considered safe or even 90 91 beneficial for health (Ehehalt et al. 2010; Mourad et al. 2010; Stremmel et al. 2010), the impact of soy 92 lecithin on the gut microbiota has never been studied. Since this compound is one of the most 93 extensively used food emulsifiers, it was incorporated in this research.

Finally, rhamnolipids and sophorolipids are two biotechnological emulsifiers of microbial origin. Due
 to their advantageous properties with respect to (eco)toxicity and waste stream reuse (Van Bogaert,

96 Zhang, and Soetaert 2011; Haba et al. 2003; Costa et al. 2017) they are currently under consideration 97 as novel food additives (Costa et al. 2017; Nitschke and Silva 2018; Cameotra and Makkar 2004). 98 Their strong emulsifying capacities (Van Bogaert, Zhang, and Soetaert 2011; Costa et al. 2017) and 99 more natural origin could qualify them as adequate alternatives for emulsifiers of chemical origin, 90 which the food industry is currently seeking to replace under the umbrella of the 'clean label' trend 91 (Asioli et al. 2017; Nitschke and Silva 2018; Costa et al. 2017). However, given their strong 92 antimicrobial properties, an evaluation at the level of the gut microbiota is highly warranted before 93 multiplication of the service of the gut microbiota is highly warranted before

103 such applications can be legalized.

Here we investigated the effects of the five above-mentioned dietary emulsifiers on human fecal microbiota through 48h *in vitro* batch incubations. This set of emulsifiers enables the comparison of the previously-studied chemical emulsifiers with the natural extract lecithin and with biosurfactants. In order to take into account interindividual variability in microbiome composition as a possible determinant of the putative impact from emulsifiers, we separately assessed microbial incubations from

- 109 ten different individuals.
- 110

<sup>&</sup>lt;sup>2</sup> FDA. 2019. "CFR - Code of Federal Regulations Title 21 - Sodium Carboxymethyl Cellulose." 2019. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=182.1745.

<sup>&</sup>lt;sup>3</sup> FDA. 2020. "CARBOXYMETHYL CELLULOSE, SODIUM SALT." 2020. https://www.accessdata.fda.gov/scripts/fdcc/?set=FoodSubstances&id=CARBOXYMETHYLCELLULOSESODIUMSALT&sort=Sortterm&order=ASC &startrow=1&type=basic&search=carboxymethyl.

<sup>&</sup>lt;sup>4</sup> European Commision. "00172 - Polysorbates -E433." 2019. Accessed August 6, 2019. https://webgate.ec.europa.eu/foods\_system/main/index.cfm?event=substance.view&identifier=172.

<sup>&</sup>lt;sup>5</sup> FDA. "CFR - Code of Federal Regulations Title 21 - Polysorbate 80." 2019. Accessed August 6, 2019. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.840.

<sup>&</sup>lt;sup>6</sup> European Commision 2018. "00115 -Soy Lecithin - E322." 2018. https://webgate.ec.europa.eu/foods system/main/index.cfm?event=substance.view&identifier=115.

<sup>&</sup>lt;sup>7</sup> FDA. 2019. "CFR - Code of Federal Regulations Title 21 - Soy Lecithin." 2019. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=184.1400.

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## 111 2 Materials and Methods

# 112 2.1 Experimental design

Fecal material from 10 human individuals was collected and separately incubated for 48h with the five emulsifiers at three concentrations (0.005% (m/v), 0.05% (m/v) and 0.5% (m/v)). Emulsifier concentrations were chosen based on the maximal legal concentration in food products (EFSA & FDA), which comply with commonly applied concentrations in food products (Msagati 2012; Mallet 1992; Adams et al. 2004). Each donor incubation series also included a control condition, in which a

118 sham treatment with an equivalent volume of distilled water was performed.

119 The emulsifiers used during this study were sodium carboxymethylcellulose (CMC), polysorbate 120 (P80), soy lecithin, sophorolipids and rhamnolipids. CMC (catalogue number 419303 - average molecular weight of 250 000 g/mol, degree of substitution of 0.9), P80 (P4780 - suitable for cell 121 122 culture) and rhamnolipids (RL) (R90 - 90% pure) were obtained from Sigma Aldrich, St. Louis, MO. 123 Soy lecithin was obtained from Barentz Unilecithin (UNILEC - ISL non GMO IP) and sophorolipids 124 were obtained from the UGent Inbio group from the Centre for Synthetic Biology. The latter were 125 described as 75% (w/v) solutions and their composition was determined to be mainly lactonic, 126 diacetylated C18:1 SL.

Donors 2 and 6 reported to follow a vegetarian and vegan diet. All other donors consumed an omnivorous diet. The age of the four female and six male donors varied from 23 to 53 years old. None of the donors received any antibiotic treatment in the 3 months prior to their donation. Experimental work with fecal microbiota from human origin was approved by the ethical committee of the Ghent University hospital under registration number BE670201836318.

## 132 **2.2 Batch incubation**

133 Before incubation, the five emulsifiers were supplemented to amber penicillin bottles containing 40

mL of autoclaved low-sugar nutritional medium (per L: 0.25 g gum arabic, 0.5 g pectin,0.25 g xylan,
1 g starch, 3 g yeast extract, 1 g proteose peptone, 2 g pig gastric mucin; all from Sigma Aldrich, St.

Louis, MO). The amounts of emulsifiers to add were calculated to obtain concentrations of 0.005%

(m/v), 0.05% (m/v) and 0.5% (m/v) in a final volume of 50 mL (the volume obtained after addition of

138 the fecal slurry). The bottles were stored in a 4°C fridge until use (for maximum 3 days).

At the start of the batch experiments, the penicillin flasks containing nutritional medium and 139 140 emulsifiers were brought to room temperature to provide an ideal growth environment for the fecal 141 bacteria. Fresh fecal samples were then collected in airtight plastic lidded containers. Anaerogen<sup>TM</sup> 142 sachets (Oxoid Ltd., Basingstoke, Hampshire, UK) were used to sequester O2. The samples were stored 143 at 4°C until use for a maximum of 3 hours. A fecal inoculum was then prepared as described in De 144 Boever, Deplancke, and Verstraete (2000), by mixing 20% (w/v) fecal material into a 0.1 M anaerobic 145 phosphate buffer (pH 6.8) supplemented with 1 g/L sodium thioglycanate as a reducing agent. Into 146 each penicillin bottle, 10 mL of fecal inoculum was added, after which the bottles were closed with 147 butyl rubber stoppers and aluminium caps. The headspaces were flushed with a N<sub>2</sub>/CO<sub>2</sub> (80/20)-gas mixture using gas exchange equipment to obtain anaerobic conditions and incubated in an IKA® KS 148 149 4000 I Control shaker at 200 rpm at 37°C. During the course of the experiments, the pH was followed 150 up every day with a Prosense QP108X pH-electrode connected to a Consort C3020 multi parameter

151 analyzer to ensure stable and viable growth conditions (pH remained within 5.5 - 6.8).

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Aliquots were taken on three timepoints: immediately after the start of the incubation (T0) (2-3 hours after combining the fecal inoculum with the medium containing emulsifier), after 24 hours of incubation (T1) and after 48 hours of incubation (T2). Samples were taken for short chain fatty acid (SCFA-) analysis (1 mL), 16S rRNA gene amplicon sequencing (1 mL), for flagellin detection (500  $\mu$ L) and for immediate fluorescent cell staining and flow cytometry (100  $\mu$ L). SCFA-, flagellin- and sequencing samples were stored at -20°C until analysis.

# 158 2.3 Intact/damaged cell counts

159 To assess the impact of the emulsifiers on total and intact cell concentrations, cell staining with SYBR® 160 green and propidium iodide was performed after which cells were counted on an Accuri C6+ Flow 161 cytometer from BD Biosciences Europe. The combination of these two cell stains is frequently used to distinguish intact bacterial cells from cells damaged at the level of the cell membrane, since SYBR® 162 green enters any cell rapidly, while propidium iodide, being a larger molecule, enters intact cells much 163 164 slower and mainly stains damaged cells within commonly applied incubation times (Van Nevel et al. 2013). Samples were analyzed immediately after sampling to preserve the intact cell community. 165 Dilutions up to 10<sup>-4</sup> and 10<sup>-5</sup> were prepared in 96-well plates using 0.22 µm filtered 0.01 M phosphate 166 buffered saline (PBS) (HPO<sub>4</sub><sup>2-</sup> / H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, 0.0027 M KCl and 0.137 M NaCl, pH 7.4, at 25 °C) and these 167 168 were subsequently stained with SYBR® green combined with propidium iodide (SGPI, 100x 169 concentrate SYBR® Green I, Invitrogen, and 50x 20 mM propidium iodide, Invitrogen, in 0.22 µm-170 filtered dimethyl sulfoxide) (Van Nevel et al. 2013; Props et al. 2016a). After 25 minutes of incubation, 171 the intact and damaged cell populations were measured immediately with the flow cytometer, which 172 was equipped with four fluorescence detectors (530/30 nm, 585/40 nm, >670 nm and 675/25 nm), two 173 scatter detectors and a 20 mW 488 nm laser. The flow cytometer was operated with Milli-Q (Merck 174 Millipore, Belgium) as sheath fluid. The blue laser (488 nm) was used for the excitation of the stains 175 and a minimum of 10,000 cells per sample were measured for accurate quantification. Settings used were an FLH-1 limit of 1000, a measurement volume of 25 µL and the measurement speed was set to 176 177 'fast'. Cell counts were obtained by gating the intact and damaged cell populations in R (version 3.6.2) 178 according to the Phenoflow-package (v1.1.6) (Props et al. 2016b). Gates were verified using data from

179 negative control samples (only 0.22 µm filtered 0.01 M PBS) (Figure S1).

# 180 **2.4 SCFA-analysis**

181 The SCFA-concentrations were determined by means of diethyl ether extraction and capillary gas 182 chromatography coupled to a flame ionization detector as described by De Paepe et al. (2017); 183 Anderson et al. (2017). Briefly, 1 mL aliquots were diluted 2x with 1 mL milli-Q water and SCFA 184 were extracted by adding approximately 400 mg NaCl, 0.5 mL concentrated H<sub>2</sub>SO<sub>4</sub>, 400 µL of 2-185 methyl hexanoic acid internal standard and 2 mL of diethyl ether before mixing for 2 min in a rotator 186 and centrifuging at 3000 g for 3 minutes. Upper layers were collected and measured using a GC-2014 187 capillary gas chromatograph (Shimadzu, Hertogenbosch, the Netherlands), equipped with a capillary 188 fatty acid-free EC-1000 Econo-Cap column (Alltech, Lexington, KY, US), 25 m × 0.53 mm; film thickness 189 1.2 µm, and coupled to a flame ionization detector and split injector. One sample (donor 9, timepoint 2 190 -0.05% (m/v) CMC) returned only zero values, presumably due to a technical error. This sample was 191 therefore omitted prior to computational analyses.

## 192 **2.5** Amplicon sequencing

193 Samples from T0 and T2 were selected for Illumina 16S rRNA gene amplicon sequencing. The samples

194 (1 mL) were first centrifuged for 5 min at 30 130 g in an Eppendorf 5430 R centrifuge to obtain a cell

195 pellet. After removing the supernatant, the pellets were subjected to DNA-extraction (Vilchez-Vargas

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- et al. 2013; De Paepe et al. 2017). The pellets were dissolved in 1 mL Tris/HCl (100 mM, pH = 8.0)
- 197 supplemented with 100 mM EDTA, 100 mM NaCl, 1% (w/v) polyvinylpyrrolidone and 2% (w/v) 198 sodium dodecyl sulfate after which 200 mg glass beads (0.11 mm Sartorius, Gottingen, Germany) were
- added and the cells were lysed for 5 min at 2000 rpm in a FastPrep VR-96 instrument (MP Biomedicals,
- Santa Ana, CA). The beads were then precipitated by centrifugation for 5 min at 30 130 g and the
- supernatant was collected. Purification of DNA took place by extraction of cellular proteins with 500
- supernatant was confected. Furtheation of DNA took place by extraction of central proteins with 500  $\mu$ L phenol-chloroform-isoamilyc alcohol 25-24-1 at pH7 and 700  $\mu$ L 100% chloroform. The DNA was
- 203 precipitated by adding 1 volume of ice-cold isopropyl alcohol and 45 µL sodium acetate and cooling
- for at least 1h at -20°C. Isopropyl alcohol was then separated from DNA by centrifugation for 30 min
- at 4°C and at 30 130 g and the pellet was dried by pouring off the supernatant. It was resuspended in
- 206 100 mL 1x TE buffer (10 mM Tris, 1 mM EDTA) for storage at  $-20^{\circ}$ C.
- 207 DNA-quality was verified by electrophoresis in a 1.5% (w/v) agarose gel (Life technologies, Madrid, 208 Spain) and DNA-concentration was determined using a QuantiFluor® dsDNA kit (detection limit: 50 209 pg/mL; sensitivity:  $0.01 - 200 \text{ ng/}\mu\text{L}$ ) and Glomax® –Multi+ system (Promega, Madison, WI) with 210 the blue fluorescence optical kit installed (Ex: 490nm, Em: 510–570 nm).
- Library preparation and next generation 16S rRNA gene amplicon sequencing were performed at the VIB nucleomics core (VIB, Gasthuisberg Campus, Leuven, Belgium) as described in Tito et al. (2017). The V4 region of the 16S rRNA gene was amplified using the bacterial 515F (GTGYCAGCMGCCGCGGTAA) and the 806R (GGACTACNVGGGTWTCTAAT) primers, which were modified with both Illumina adapters as well as adapters for directional sequencing. Sequencing was then performed on an Illumina MiSeq platform (Illumina, Hayward, CA, USA) according to manufacturer's guidelines.
- One sample (donor 3, timepoint 2, 0.05% (m/v) sophorolipids) failed to sequence. The sequencing data
   have been submitted to the National Center for Biotechnology Information (NCBI) database under the
   accession number PRJNA630547.
- 221 Processing of amplicon data was carried out using mothur software version 1.40.5 and guidelines 222 (Kozich et al. 2013). First, contigs were assembled, resulting in 14 977 727 sequences, and ambiguous 223 base calls were removed. Sequences with a length of 291 or 292 nucleotides were then aligned to the 224 silva\_seed nr.123 database, trimmed between position 11 895 and 25 318 (Quast et al. 2013). After 225 removing sequences containing homopolymers longer than 9 base pairs, 92% of the sequences were 226 retained resulting in 2 957 626 unique sequences. A preclustering step was then performed, allowing 227 only three differences between sequences clustered together and chimera.vsearch was used to remove 228 chimeras, retaining 79% of the sequences. The sequences were then classified using a naïve Bayesian 229 classifier against the Ribosomal Database Project (RDP) 16S rRNA gene training set version 16, with 230 a cut-off of 85% for the pseudobootstrap confidence score. Sequences that were classified as Archaea, 231 Eukaryota, Chloroplasts, unknown or Mitochondria at the kingdom level were removed. Finally, 232 sequences were split at the order level into taxonomic groups using the opticlust method with a cut-off 233 of 0.03. The data was classified at a 3% dissimilarity level into OTUs resulting in a .shared (count
- table) and a .tax file (taxonomic classification).
- For the entire dataset of 319 samples, 95 511 OTUs were detected in 175 genera. An OTU was in this manuscript defined as a collection of sequences with a length between 291 and 292 nucleotides and with 97% or more similarity to each other in the V4 region of their 16S rRNA gene after applying hierarchical clustering.
- 238 m 239

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# 240 **2.6** Cell culture for flagellin detection

241 Murine TLR5-expressing HEK 293 cells (InvivoGen), which are designed to respond to bacterial 242 flagellin in cell culture medium, were cultured according to the manufacturer's guidelines. Cells were 243 grown from an in house created frozen stock in Dulbecco's modified Eagle's (DMEM) growth medium (4.5 g/L glucose, 10% fetal bovine serum, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-244 glutamine) supplemented with 100 µg/mL Normocin<sup>TM</sup> and maintained in culture in DMEM growth 245 medium supplemented with 100  $\mu$ g/mL Normocin<sup>TM</sup>, 10  $\mu$ g/mL of blasticidin and 100  $\mu$ g/mL of 246 zeocin<sup>TM</sup>. Medium was refreshed every two days and cells were passaged when reaching 70-80% 247 248 confluency.

- 249 Assays for flagellin detection were performed as instructed by Invivogen, using cells from passage 4-250 9. Samples from donor 3, 5 and 7 were selected for this assay based on a high, intermediate and low 251 metabolic response to the emulsifiers, as measured by the SCFA-levels. Before combining with the 252 HEK-blue cells, the samples were purified to obtain only the bacterial cells by first diluting them 1/4 in 253 UltraPure<sup>TM</sup> DNase/RNase-Free distilled water (Invitrogen), then centrifuging twice at 4 226 g for 10 254 min, with a washing step using 0.22 µm filtered -/- PBS in between. The resulting cell pellet was 255 dissolved into 0.22  $\mu$ m filtered -/- PBS. A standard curve (125 ng/mL – 1.95 ng/mL), prepared from 256 recombinant flagellin from Salmonella typhymurium (RecFLA-ST, Invivogen) in sterile water was also 257 added to the plate in triplicate. After an incubation for 23h, absorbances were obtained using a Tecan 258 Infinite F50 plate reader at 620 nm.
- 259 As a check for the viability of the cell culture after combination with the samples, a resazurin assay 260 was performed. To this end, the supernatant from the cell culture plate used for the flagellin assay was 261 discarded after the first incubation phase. The cells were then washed using 0.22 µm filtered -/- PBS. 262 As a positive control, 3 wells were spiked with 20  $\mu$ L of a 5% Triton solution. The rest of the wells 263 received 20 µL -/- PBS after which 180 µL of a of 0,01 mg/mL resazurin solution was added to all 264 wells. After 3 hours of incubation at 37°C and 10% CO<sub>2</sub>, cell activity was measured using a Glomax® 265 -Multi1 system (Promega, Madison, WI) with filter the green fluorescence optical kit (Ex: 525 nm, Em: 580-640 nm). 266

## 267 2.7 Data analysis and statistics

268 Data visualization and processing was performed in R version 3.4.2 (2017-09-28) (R Core Team, 2016) 269 and Excel 2016. All hypothesis testing was done based on a significance level of 5% ( $\alpha = 0.05$ ).

# 270 2.7.1 Cell counts and SCFA

After loading the cell count table in R, total cell counts were calculated as the sum of the intact and damaged cell counts. The data were explored by calculating intact/damaged cell count ratio's and percentages of intact cells at different timepoints. For plotting, both the 10 000x and 100 000x dilutions were taken into account. Boxplots of total and intact cell counts, as well as intact/damaged ratio's were created using ggplot2 (v3.2.1) in which the stat\_compare\_means function was used to check the significance of the emulsifier effect, by means of a Kruskal-Wallis test.

Statistical analysis of SCFA-levels was similar to that of the cell counts. Production levels of acetate, propionate and butyrate over  $48h (C_{T2}-C_{T0})$  were first calculated and then boxplots were created using ggplot2. Significance of the effect of the emulsifier treatment was tested with stat\_compare\_means

- using Wilcoxon Rank Sum tests with Holm correction and Kruskal-Wallis test for overall group
- comparison.

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# 282 **2.7.2 Amplicon sequencing data**

283 The shared and taxonomy files resulting from the mothur pipeline were loaded into R for further 284 processing. Singletons (OTUs occurring only once over all samples) were removed, resulting in 36 496 285 OTUs being retained (McMurdie and Holmes 2014). Rarefaction curves were created to evaluate the 286 sequencing depth (Figure S2) (Oksanen et al. n.d.). As the number of 16S rRNA gene copies present 287 within bacteria differs between species, a copy number correction of the reads was carried out by first 288 classifying the representative sequences of the OTUs (also obtained via the mothur pipeline) using the 289 online RDP classifier tool, then obtaining both a copy number corrected read classification and a non 290 copy number corrected one, calculating the copy number by dividing both and finally dividing the 291 acquired read counts in the shared file by the calculated copy numbers.

Both relative and absolute abundances of the OTUs and genera were calculated from the copy number corrected read counts and were explored via bar plots using ggplot2 (v3.2.1). Relative abundances were calculated as percentages of the total read counts per sample. Absolute abundances were calculated (Quantitative microbial profiling) by multiplying the total cell counts obtained via flow cytometry with the relative abundances of the OTUs (similar to Vandeputte et al. 2017).

297 Overall community composition was visualized using Principle Coordinate Analysis (PcoA) on the 298 abundance based Jaccard distance matrix using the cmdscale-function in the stats (v3.6.2) package. To investigate the effects of the individual contraints on the microbial community a series of distance 299 300 based redundancy analyses (dbRDA) was then performed on the scores obtained in the PCoA on the 301 Jaccard distance matrix using the capscale function in the vegan (v2.5-6) package. Permutation tests 302 were used to evaluate the significance of the models and of the explanatory variables (De Paepe et al. 303 2018). The global model included the factors Emulsifier, Emulsifier concentration, Timepoint and 304 Donor as explanatory variables and the absolute abundances of the genera as explanatory variables. In 305 a first dbRDA this full model was included, to investigate the share of variance explained by each 306 constraint variable. The timepoint factor was distinguished as the factor causing the largest share of 307 variance and since its effect was of little interest to us it was partialled out in further dbRDAs. To check 308 for the effect of the donor variable on the microbial community, a second and third dbRDA were 309 performed, with and without conditioning of the donor variable. The final model then visualized the 310 effects of the treatments (defined by factors emulsifier and emulsifier concentration). The results of the 311 dbRDAs were plotted as type II scaling correlation triplots showing the two first constrained canonical 312 axes (labelled as dbRDA Dim 1/2) and the proportional constrained eigenvalues representing their 313 contribution to the total (both constrained and unconstrained) variance.

The chao1, chaobunge2002, ACE-1, Shannon, Simpson, InvSimpson and Pielou diversity indices were calculated for the microbial community after 48h incubation based on the copy number corrected OTUtable using the SPECIES (v1.0) package and the diversity function in the vegan (v2.5-6) package. Indices were plotted using ggplot2 and significances were tested using pairwise Wilcoxon Rank Sum

tests with Holm correction (ggpubr package v.0.2.4).

To evaluate differential abundance of genera between emulsifier treatments and control, the DESeq2 package (v 1.24.0) was applied on the copy number corrected count-table at genus level. In order to streamline the DESeq-analysis, pre-filtering according to McMurdie and Holmes 2014 was first applied on the copy number corrected count-table, after which a genus-level table was created using the aggregate function (stats package v3.6.3). In the generalized linear model, the factor Timepoint, Donor and Treatment – a concatenation of the emulsifiers and their concentrations – were included. A likelihood ratio test was employed within the DESeq function on the reduced model, containing only bioRxiv preprint doi: https://doi.org/10.1101/2020.06.28.174946; this version posted June 28, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made **Dietary emulsifiers affect give interview** -BY-NC-ND 4.0 International license.

326 the factors Donor and Timepoint, to test for the significance of the model. Low count genera were 327 subjected to an empirical Bayesian correction (Love, Huber, and Anders 2014). For pairwise 328 comparison of treatments versus controls, Wald tests were used after shrinkage of the Log2FoldChange 329 values by means of the lfcShrink function. P-values were adjusted by means of a Benjamini-Hochberg 330 procedure (Love, Huber, and Anders 2014). Results were visualized in volcanoplots, displaying the – 331 log(adjusted p-value) versus the Log2FoldChange of each genus. Additionally, box plots were created 332 showing the log-transformed pseudocounts extracted by the plotCounts function for each genus that 333 showed significant differential abundance. Since for CMC and P80 no significantly altered genera were

found, these emulsifiers were omitted from the boxplots.

335 Finally, to summarize relations between the emulsifier treatments and the intact cell counts, the SCFA-336 data and the 16S rRNA sequencing data, a partial redundancy analysis was carried out performed using 337 the rda function in the vegan package (v2.5.6). The acetate, propionate and butyrate concentrations, 338 the intact cell counts and the relative abundance of the genera as response variables and the factors 339 Emulsifier, Emulsifier concentration, Donor and Timepoint as explanatory variables. Since the 340 response variables carried different units, they were first centered around their mean using the scale 341 function (base R v3.6.2). The factors Donor and Timepoint were partialled out to visualize solely the 342 effect of the emulsifier treatments. The statistical significance of the effects was tested via a 343 permutation tests and the results were plotted in a type II correlation triplot showing the first two 344 constrained canonical axes (RDA1/2) annotated with their proportional eigenvalues representing their 345 contribution to the constrained variance. The sites were calculated as weighed sums of the scores of 346 the response variables.

## 347 2.7.3 Metagenome prediction

348 Indications of functionality from phylogenetic information were obtained using PICRUSt 349 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al. 350 2013). An OTU-table was first generated against the Greengenes reference database (v13.8) using a 351 closed ref OTU-picking strategy. The obtained OTU-table was then run through PICRUSt's 352 normalize by copy number.py script (Langille et al. 2013), which divides the abundance of each OTU 353 by its inferred 16S copy number (the copy number is inferred from the closest genome representative 354 for a 16S Greengenes reference sequence). The metagenome was then predicted using Kyoto 355 Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2012). The prediction 356 provided an annotated table of predicted gene family counts for each sample, where gene families were 357 grouped by KEGG Orthology identifiers. Significantly different L2-level pathways across emulsifier concentration were visualized in boxplots using the ggplot2 package (v3.2.1). Kruskal-Wallis tests 358 359 were performed for overall comparison of emulsifier concentrations within L2-pathways for each 360 emulsifier and Wilcoxon rank sum tests were used for pairwise comparison of emulsifier 361 concentrations vs control. Also relying on PICRUSt (Langille et al. 2013), the BugBase tool (Ward et 362 al. 2017) was used to determine the relative degree of biofilm formation, oxygen utilization, pathogenic 363 potential, oxidative stress tolerance and Gram-stain of the bacteria in the samples.

## 364 2.7.4 Flagellin concentration

365 Initial data processing was executed in Excel 2016. First, a four parametric logistic model was fitted 366 to the standard curve using the 4PL-Curve Calculator from aatbio.com (AAT Bioquest n.d.). Given our 367 observation that the emulsifiers decreased HEK cell activity, flagellin concentrations were normalized 368 by use of absorbance values obtained from the resazurin assays: flagellin concentrations were divided 369 by the ratio of the absorbance values from the samples over the average absorbance values for the 370 standard curve of the same plate. Graphs were created using ggplot2. Since donors were observed bioRxiv preprint doi: https://doi.org/10.1101/2020.06.28.174946; this version posted June 28, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made **Dietary emulsifiers affect give interview** -BY-NC-ND 4.0 International license.

371 separately and no replicate experiments per donor were performed, no statistical tests were performed

372 for the flagellin data.

# 373 2.8 Donor diversity analysis

We sought to assess the overall susceptibility of the 10 donors to the effects of the emulsifiers. Since literature describes no workflow for this purpose, we elaborated our own. Donors were ranked in terms of their susceptibility to the emulsifiers using several parameters: The 48h production of the three most abundant SCFA (acetate, propionate and butyrate), the intact cell counts at T2 and the relative and absolute abundance of the most abundant OTU in the OTU -table, *Escherichia/Shigella*, at T2. These calculations were performed in Excel 2016.

First, to correct for the batch-effect, the control-values were subtracted from the treatment-values for each donor. Next the corrected treatment values were summated for each donor, to obtain a single value that expressed the donor's susceptibility and these values were then used to rank the donors from least to most susceptible for every parameter, visualized in bar graphs. This workflow was followed for each parameter.

## 385 2.9 Comparison of equivalent emulsifier concentrations

386 Due to their stronger emulsifying properties, rhamnolipids and sophorolipids could reportedly be used 387 in lower concentrations in food products than conventional chemical emulsifiers (Nitschke and Silva 388 2018). Therefore, we sought to compare the effects of the chemical emulsifiers, CMC and P80, versus 389 those of the biosurfactants, rhamnolipids and sophorolipids, with regards to their impacts on the gut 390 microbiota at equivalent emulsifying concentrations. Wilcoxon Rank Sum test were executed in R 391 using the compare\_means function on the same parameters we used to evaluate donor diversity (see 392 3.7). As equivalent concentrations we considered a 10x lower concentration of biosurfactants compared 393 to the chemical emulsifiers, given that this is what industry reports (Van Haesendonck and Vanzeveren 394 2006). Hence we compared the condition of 0.5% (m/v) of chemical emulsifiers with that of 0.05% 395 (m/v) of biosurfactancs and 0.05% (m/v) of chemical emulsifiers with 0.005% (m/v) of biosurfactants.

396 **3 Results** 

# **397 3.1 Community structure is altered by addition of dietary emulsifiers.**

## 398 **3.1.1 Intact/damaged cell counts**

399 Analysis of intact and damaged cell populations with flow cytometry (SGPI-staining defines damage 400 at the level of the cell membrane (Wlodkovic, Skommer, and Darzynkiewicz 2009; Buysschaert et al. 401 2018; Falcioni, Papa, and Gasol 2008), was used as a proxy for emulsifier toxicity. First, total and 402 intact cell counts in the controls dropped by  $14\% \pm 2\%$  and  $21\% \pm 3\%$ , respectively, after 48h in vitro 403 incubation (Table S1 and S2). When considering the impact of the emulsifiers, we observed that higher 404 concentrations of rhamnolipids, sophorolipids and soy lecithin resulted in significantly lower total and 405 intact cell counts (Figure 1 and 2). At 0.5% (m/v) rhamnolipids, intact cells decreased by 91%  $\pm$  0% 406 after 48h compared to the control sample of T0. At 0.5% (m/v) sophorolipids, about 89%  $\pm$  1%, was 407 lost and at 0.5% (m/v) soy lecithin,  $87\% \pm 1\%$  was lost. The toxic effects were immediate for the 408 sophorolipids and rhamnolipids, while for soy lecithin this decreasing effect only became significantly 409 apparent after 24h (T1) (Figure 2). The impact of CMC and P80 towards the cell population was less 410 pronounced. CMC even increased the total cell counts (not significantly) at higher concentrations, 411 although the fraction of living cells remained unaffected for all CMC-conditions.

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## 412 **3.1.2 Microbial community**

The impact from the emulsifiers towards microbial community structure was assessed with 16S rRNA gene amplicon sequencing. First, the *in vitro* conditions had an impact on microbiota composition. While each donor showed a unique profile of microbial genera at the start of the experiment more similar microbial community profiles were obtained upon incubation, primarily due to an increase in *Escherichia/Shigella* abundance from  $0.02\% \pm 0.02\%$  to  $16\% \pm 25\%$  (Fig S3 – S5).

418 Independent from the in vitro effects, clear differences were noted between emulsifier treatments and 419 controls, which were both emulsifier- and donor-dependent (Figure 3, Figure S4 and S5). Where the 420 effects of rhamnolipids and sophorolipids were most outspoken, the impact of soy lecithin was 421 intermediary and CMC and P80 had the smallest impacts (Figure 1). This was evidenced by significant 422 drops in diversity indices upon incubation with rhamnolipids, sophorolipids and to a lesser extent soy 423 lecithin (Figure 4). DESeq-analysis further revealed significant differential relative abundance of 36 424 genera, from which 23 were increased and 13 were suppressed, compared to the control condition 425 (Figure 5 and 6). Rhamnolipids triggered the strongest changes, with the three most increased genera 426 being unclassified *Enterobacteriaceae* (L2FC = 3.85; Padj <0.0001), *Fusobacterium* (L2FC = 2.75; 427 Padj < 0.0001) and *Escherichia/Shigella* (L2FC = 2.49; Padj < 0.0001) and the three most suppressed 428 ones being unclassified *Bacteroidetes* (L2FC = -2.19; Padj = < 6.323E-4), *Barnesiella* (L2FC = -2.09; 429 Padj <0,009) and *Bacteroides* (L2FC = -2.02; Padj <0,002). The top three most increased genera for 430 sophorolipids were *Escherichia/Shigella* (L2FC = 1.86; Padj <0,043), *Acidaminococcus* (L2FC = 1.80; 431 Padj < 0.0001) and *Phascolarctobacterium* (L2FC = 1.68; Padj = < 0.0001) and the three most decreased 432 were unclassified Bacteroidetes (L2FC = -1.97; Padj <0.0001), Barnesiella (L2FC = -1.70; Padj = 433 <0.0001) and *Bacteroides* (L2FC = -1.53; Padj = 3,034E-06). The top three most increased genera by soy lecithin were Acidaminococcus (L2FC = 1.23; Padj =0.016), Porphyromonadaceae unclassified 434 435 (L2FC = 1.19; Padj = 0.017) and *Sutterella* (L2FC = 1.19; Padj = 0.004). Two significantly decreased 436 genera were *Flavonifractor* (L2FC = -1.04; Padj = 0.009) and *Pseudoflavonifractor* (L2FC = -0.95; 437 Padj = 0.015) (Figure 5 and 6).

## 438 **3.2 Functional analysis**

## 439 **3.2.1 SCFA**

440 Short chain fatty acids were analyzed to study how exposure to dietary emulsifiers affects the general 441 microbial metabolic activity. We observed that SCFA-production was significantly and differently 442 affected by rhamnolipids, sophorolipids and soy lecithin, while no changes were observed for P80 and 443 CMC (Figure 1 and 7). The strongest impacts were noted for rhamnolipids, which, at 0.5% (m/v), 444 significantly decreased total SCFA production by about  $36\% \pm 5\%$  (P<sub>wilcox</sub> <0.0001) compared to the 445 control condition. This decrease was mainly attributed to a  $32\% \pm 7\%$  decrease in acetate production 446 (Pwilcox <0.0001) compared to the control. Rhamnolipids at 0.5% (m/v) also reduced butyrate 447 production by 96%  $\pm$  6% compared to the control condition (P<sub>wilcox</sub> <0.0001) while propionate 448 production remained unaffected. Interestingly, incubation with 0.5% (m/v) sophorolipids also resulted 449 in a decrease in butyrate production by 73%  $\pm$  24% compared to the control (P<sub>Wilcox</sub> <0.0001), while 450 propionate production increased by 88%  $\pm$  50% (P<sub>Wilcox</sub> = 2.1 e-04). Soy lecithin at 0.5% (m/v) significantly increased propionate production by 29%  $\pm$  18% on average (P<sub>wilcox</sub> = 0.0089) and 451 decreased butyrate production non-significantly by  $34\% \pm 25\%$  on average (P<sub>wilcox</sub> = 0.035). No 452 453 profound shifts in microbial fermentation activity were observed for incubations with CMC and P80.

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# 454 **3.2.2 Metagenomic prediction**

Other emulsifier related functional shifts were explored via metagenomic prediction using PICRUSt. These analyses predicted suppressing effects of rhamnolipids, sophorolipids on the pathways "Biosynthesis of other secondary metabolites", "Cell growth and death" and "Signalling molecules and interaction" (Figure S6). Possible significantly upregulated L2-pathways were "Cell motility", "Cellular Processes and signalling", "Genetic information processing", "Lipid metabolism", Membrane transport", "Metabolism", "Signal transduction", "Transcription" and "Xenobiotics degradation".

462 Plugging the data into the Bugbase-webtool revealed a significantly stimulating effect of sophorolipids 463 and rhamnolipids on the formation of biofilms and mobile elements, stress tolerance and increased 464 abundance of potential pathogens, gram negative and facultative anaerobic bacteria all properties 465 related to the Proteobacteria phylum. This coincides with our observation of an increased abundance 466 of *Escherichia/Shigella* (Supplementary Data 1).

# 467 **3.2.3 Flagellin levels**

In order to validate the PICRUSt prediction of a higher motility potential a HEK-blue mTLR5 reporter
 cell assay was used for the detection of bacterial flagellin. The response of the flagellin concentrations

470 to the emulsifiers was found to be largely donor-dependent and inconsistent shifts were observed in

471 function of incubation time (Figure 8). Shifts in flagellin levels upon emulsifier dosage were variable,

472 meaning that the prediction of higher motility by PICRUSt could not be substantiated.

## 473 **3.3 Donor Diversity**

For all endpoints, inter-individual variability was observed in response to the *in vitro* incubations and emulsifier treatments. In terms of community structure, Figure 3A shows that each donor clusters separately. This clustering was found to be significant ( $P_{dbRDA} < 0.05$ ).

To assess in more detail whether there was coherence in the read-outs with respect to donor
susceptibility, we ranked the donors according to their response on the most relevant parameters, *i.e.*intact cell counts, production of the most important SCFA (acetate, propionate and butyrate) and the
absolute and relative abundance of *Escherichia/Shigella*.

We observed that the susceptibility of the donors to the effects of the emulsifiers depended on the targeted parameter (Figure 9). Some donors consistently ranked as highly susceptible (D9) or less susceptible (D6 and D1), while for other donors, ranking was more variable over the different parameters.

## 485 **3.4 Equivalent emulsifier concentrations**

486 We sought to compare the effects of biosurfactants *versus* those of conventional chemical emulsifiers. 487 When comparing 0.5% (m/v) of chemical emulsifier with 0.05% (m/v) of biosurfactant – the 488 concentrations most representative of the currently applied levels of dietary emulsifiers - the 489 previously described effects of the biosurfactants (decreased levels of acetate, butyrate, intact and total cell concentration and increased abundance of propionate and increased abundance 490 of 491 *Escherichia/Shigella*) were significant compared to the effects of the chemical emulsifiers (Table S3). 492 When comparing 0.05% (m/v) of chemical emulsifier with 0.005% (m/v) of biosurfactant, the effects 493 were not significantly different, except for the effects of rhamnolipids on the cell count data.

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#### 494 **4 Discussion**

495 We found dietary emulsifiers to significantly alter human gut microbiota towards a composition and 496 functionality with potentially higher pro-inflammatory properties. While donor-dependent differences 497 in microbiota response were observed, our in vitro experimental setup showed these effects to be 498 primarily emulsifier-dependent. Rhamnolipids and sophorolipids had the strongest impact with a sharp 499 decrease in intact cell counts, an increased abundance in potentially pathogenic genera like 500 Escherichia/Shigella and Fusobacterium, a decreased abundance of beneficial Bacteroidetes and 501 Barnesiella and a predicted increase in flagellar assembly and general motility. The latter was not 502 substantiated through direct measurements, though. The effects were less pronounced for soy lecithin, 503 while chemical emulsifiers P80 and CMC showed the smallest effects. Short chain fatty acid 504 production, with butyrate production in particular, was also affected by the respective emulsifiers, 505 again in an emulsifier- and donor-dependent manner.

- 506 One of the most profound impacts of emulsifier treatment towards gut microbiota was the decline in 507 intact microbial cell counts. The degree of microbiome elimination in this study seems comparable to 508 what has been observed for antibiotic treatments (Guirro et al. 2019; Francino 2016). Since antibiotics 509 are considered detrimental for gut ecology, this may serve as a warning sign with respect to emulsifier 510 usage. Emulsifiers also act as surfactants, which are known for their membrane solubilizing properties 511 (Jones 1999). The fact that the observed decline in microbial viability was dependent on emulsifier 512 dose and on the emulsifying potential of the supplemented compound, as measured by the aqueous 513 surface tension reduction (Table 1), leads us to conclude that the dietary emulsifiers attack the bacterial
- 514 cells principally at the level of the cell membrane.
- 515 We also found this antimicrobial effect from the tested emulsifiers to be highly selective, confirming 516 previous observations by Moore (1997) who showed that the effects of surfactants are dependent on 517 the bacterial species. We found the Escherichia/Shigella genus to be particularly resistant against the 518 surfactants antimicrobial effects. This agrees with Kramer et al. (1984) and Nickerson and Aspedon 519 (1992) who demonstrated the surfactant resistance of enteric bacteria Enterobacter cloaca and E. coli 520 against sodium dodecyl sulphate. They showed that this resistance is widespread among 521 Enterobacteriaceae, that it's energy-dependent and that exposure to sodium dodecyl sulphate altered 522 the expression of 19 proteins, of which three were later tentatively identified as clpP, clpB and clpX 523 intracellular proteases (Rajagopal, Sudarsan, and Nickerson 2002). Also membrane-derived 524 oligosaccharides, present in the periplasm of gram negative bacteria, were found to be essential to the 525 detergent-resistant properties of E. coli (Rajagopal et al. 2003). Even though this could explain the 526 increased abundance in this study for *Enterobacteriaceae* and *Escherichia/Shigella*, the mechanism by 527 which other species are (un)affected by the emulsifiers is not known.
- 528 Interestingly, our prediction of altered functionality using PICRUSt analysis indicated a potential 529 increase in levels of motility-genes, even though we could not confirm increased flagellin levels by 530 direct measurement. Increased motility has been observed upon addition of emulsifiers before (Lock 531 et al. 2018). This finding may again be related to the increased relative abundance of 532 Escherichia/Shigella, since these are known flagella (gene)-bearers (Mittal et al. 2003; Tominaga et al. 533 2001; Girón 1995). Increased flagellin levels constitute a potential health risk as flagellin is considered 534 an important virulence factor: this particular microbe-associated molecular pattern may trigger 535 inflammation upon binding to TLR5. A higher degree of flagellation would thus represent more motile 536 bacteria and result in a gut microbiome that is able to more aggressively penetrate the mucus layer and 537 subsequently reach gut epithelial cells (Chassaing et al. 2015; Ramos, Rumbo, and Sirard 2004). 538 Chassaing et al. (2015; 2017) demonstrated possible consequences for the host: an increased 539 inflammatory response in both gut and body, contributing to increased adiposity and weight gain.

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540 Effects towards microbial metabolic functionality, as measured by SCFA-levels, were emulsifier-541 dependent. Nevertheless, consistent shifts in SCFA-profiles were observed for all three emulsifiers for 542 which significant alterations were visible, *i.e.* a decrease in butyrate and an increase in propionate 543 production. For sophorolipids, the strong increase in propionate production can be related to the 544 increased abundance of *Phascolarctobacterium*. This genus is known to produce propionate from 545 succinate produced by E. coli cells (Del Dot, Osawa, and Stackebrandt 1993). For rhamnolipids, we 546 assume that similar cross-feeding interactions occurred, even though propionate levels remained stable. 547 We hypothesize that an increased propionate production by the Escherichia/Phascolarctobacterium 548 consortium is counteracted by the higher antimicrobial activities from the rhamnolipids at increasing 549 concentrations. The fact that L-rhamnose is a well-known propionate precursor (Reichardt et al. 2014) 550 and that rhamnolytic pathways have been observed in E. coli and other Gammaproteobacteria 551 (Rodionova et al. 2013) further support this idea. The increase in acetate and propionate production 552 observed with soy lecithin can be linked to the metabolism of glycerol (De Weirdt et al. 2010). This is 553 in agreement with the upregulated abundances of the Enterococcus and Clostridia genera at higher 554 levels of soy lecithin, since these genera are known to metabolize glycerol (Bradbeer 1965; Bizzini et 555 al. 2010) as well as choline (Martínez-del Campo et al. 2015). Also the increased abundance of the 556 Acidaminoccus genus would correspond with the increased acetate production (Chang et al. 2010). 557 These findings indicate that observed shifts in SCFA-production can be attributed to shifts in microbial 558 composition.

559 Whether the altered SCFA-production and -levels are positive or negative for host health is ambiguous. 560 On the one hand, decreased butyrate levels can be considered negative, since butyrate is known to 561 protect the gut epithelium from inflammation and cancerous growth (Liu et al. 2018; Cani 2017; Canani 562 et al. 2011). On the other hand, propionate is also considered a health-promoting SCFA (Hosseini et 563 al. 2011; Weitkunat et al. 2016; Louis and Flint 2017). Propionate production is related to decreased 564 lipogenesis in the liver and is supposed to enhance satiety mechanisms, which would lower the chance 565 of developing obesity (Hosseini et al. 2011; Weitkunat et al. 2016). It is, however, questionable whether 566 such benefit may predominate the purported negative effects from the observed antimicrobial effects 567 and the increase in pro-inflammatory markers, such as flagellated microbiota and a drop in butyrate 568 production.

569 Another point relating to health effects entails that rhamnolipids, sophorolipids and – to a lesser extent 570 - soy lecithin significantly decreased diversity parameters. Decreased microbiome diversity frequently 571 occurs with compromised health conditions such as obesity, insulin resistance, dyslipideamia 572 (Turnbaugh et al. 2009; Le Chatelier et al. 2013), type 1 diabetes (Patterson et al. 2016), heart failure 573 (Luedde et al. 2017) and inflammatory bowel disease (Arumugam et al. 2011; Benoit Chassaing et al. 574 2015). The increased prevalence of the *Escherichia/Shigella* genus has also been observed with 575 multiple metabolic conditions (Shin, Whon, and Bae 2015; Lecomte et al. 2015; Lupp et al. 2007). 576 Increased *Enterobacteriaceae* abundance was previously linked with an increased gut permeability 577 (Pedersen et al. 2018), the consumption of high fat diets (Fei and Zhao 2013; He et al. 2018; Lecomte 578 et al. 2015), colitis (Lupp et al. 2007), cardiovascular disease (Jie et al. 2017), diabetes (Deschasaux et 579 al. 2019; Allin, Nielsen, and Pedersen 2015) and even undernourishment and iron deficiency anemia 580 (Shin, Whon, and Bae 2015; Muleviciene et al. 2018). With respect to soy lecithin, metabolism of 581 phosphatidylcholine by the gut microbiota has been linked to cardiovascular disease (Wang et al. 2011; 582 Tang and Hazen 2014). We also found an increased abundance of the genus Sutterella, a bacterium 583 that has been linked to autism spectrum disorders (Wang et al. 2013) and gut inflammation, notably by 584 IgA-degradation (Kaakoush 2020; Moon et al. 2015). Overall, we can thus conclude that dietary 585 emulsifier consumption may result in profound microbiota shifts, putatively contributing to adverse 586 health outcomes.

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587 An important consideration is whether the observed in vitro effects would also take place in an in vivo setting. This will depend on a number of diet- and host-related factors. First, we found that the observed 588 589 effects were concentration- and emulsifier-dependent. Choosing an emulsifier-concentration 590 combination that minimizes adverse microbial impacts, without compromising food technical 591 properties, could thus be a strategy to mitigate the harmful effects of food emulsifiers. Second, 592 emulsifier concentration will continuously alter during gastrointestinal digestion, but the impact of the 593 dilution with accompanying food products, excretion of digestive fluids or absorption of water from 594 the gut lumen on the final concentration reaching the gut microbiota has so far not yet been studied. 595 Third, digestion by human enzymes will alter chemical structure of the emulsifiers. While CMC is 596 resistant to breakdown by human digestive enzymes (Joint FAO/WHO Expert Committee on Food 597 Additives 1973), P80 and soy lecithin can be hydrolyzed by pancreatic lipases. For P80, only the 598 polyethylene-sorbitan unit may reach the colon (Aguilar et al. 2015). Soy lecithin is mostly absorbed 599 as lysolecithin and free fatty acids, but its detection in faeces indicates that some fraction reaches the 600 colon (Mortensen et al. 2017), where the choline and glycerol moieties are metabolized by the gut 601 microbiota (Tang and Hazen 2014). For both of these compounds, it will thus be necessary to verify if 602 and to what extent the observed effects occur in vivo as well. With respect to rhamnolipids and 603 sophorolipids, no literature is currently available on their digestion by human enzymes. Preliminary 604 data from our side, however, have indicated only one alteration, a deacetylation of the sophorose units 605 of the sophorolipids (supplementary data 2). These compounds may thus readily reach the colon and interact with the endogenous microbiota. Finally, other dietary constituents in the gut, primarily lipids 606 and bile salts, will interact with emulsifiers (Naso et al. 2019). The mucus layer overlying the gut 607 epithelium and the pH-fluctuations throughout the gut are other elements that may affect emulsifier-608 microbiota interactions. 609

A last important element in the putative health impact from dietary emulsifiers concerns interindividual variability. An individual's unique microbiota and metabolism are important determinants of the potential health effects dietary emulsifiers could cause. While the overall effects from the different emulsifiers towards microbiota composition and functionality were quite consistent in our study, important interindividual differences in susceptibility of the microbiota were noted. Understanding what underlying factors and determinants drive this interindividual variability will be crucial to future health risk assessment of novel and existing dietary emulsifiers.

617 Food additives have come under scrutiny with respect to their impact on human health. Additives like 618 colorants, artificial sweeteners, nitrites (NaNO<sub>2</sub>) and high fructose corn syrup have been associated with hyperactivity, cancer development, gastric cancer and obesity, respectively. (Arnold, Lofthouse, 619 620 and Hurt 2012; Carocho et al. 2014; Bryan et al. 2012; Payne, Chassard, and Lacroix 2012). In answer 621 to these public concerns, a clean label movement has developed in the food industry that aims to 622 provide food products with a more natural image. In this light, we investigated whether rhamnolipids 623 and sophorolipids, two biotechnological emulsifiers, would yield less of a detrimental impact on the gut microbiota than the mainstream chemical emulsifiers, CMC and P80. Our results showed however, 624 625 that rhamnolipids and sophorolipids, of all emulsifiers in this study, had the strongest impact on 626 microbiota composition and functionality, even when equivalent concentrations were taken into 627 account. Further analysis of our data showed that the observed detrimental effects towards the 628 microbiota can potentially be linked to their higher emulsifying potential. All of this indicates that 629 rhamnolipids and sophorolipids are probably no appropriate alternatives to conventional emulsifiers unless they are used at substantially lower concentrations. More research must point out whether the 630 631 effects prevail *in vivo* and whether concentrations can be kept low enough to avoid adverse health 632 effects.

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#### 633 5 Figures



#### 634

Figure 1: Type II scaling triplot obtained using partial redundancy analysis of the microbial community detected after 48h of in vitro batch incubations of fecal material from 10 human donors in sugar depleted medium supplemented with 5 emulsifiers at 4 concentrations. In the main figure, the intact cell count, the SCFA-levels and the relative abundance of the top two genera are shown as response variables (red arrows) and emulsifier and emulsifier concentration are given as explanatory variables (blue arrows). The top right figure also displays the samples as sites.

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Figure 2: Average total (A) and intact (B) bacterial cell counts (cells/mL) detected during in vitro batch incubations of fecal material from 10 donors with sugar depleted medium supplemented with 5 emulsifiers at 4 concentrations. Samples were taken upon incubation (T0; 2-3h after inoculation) as well as after 24h (T1) and 48h (T2) of incubation. Asterisks indicate significant differences detected with a Kruskal Wallis test ( $\alpha = 0.05$ ).

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650 Figure 3: Type II scaling triplots obtained using partial distance based redundancy analysis of the 651 microbial community composition detected using 16S rRNA gene amplicon sequencing after 48h of in vitro batch incubations of fecal material from 10 donors with sugar depleted medium supplemented 652 with 5 emulsifiers at 4 concentrations. Samples were taken upon incubation (T0; 2-3h after inoculation) 653 654 as well as after 24h (T1) and 48h (T2) of incubation. Factors Donor, Emulsifier and Emulsifier concentration were set as explanatory variables (blue arrows) and absolute abundances of genera as 655 656 response variables (red arrows). Only the top five genera were displayed for adequate visibility. A: The factor timepoint was partialled out. B: The factors donor and timepoint were partialled out. 657

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660 Figure 4: Diversity parameters of gut microbial community obtained after 48h of in vitro batch 661 incubations of fecal material from 10 donors with sugar depleted medium supplemented with 5 662 emulsifiers at 4 concentrations. Samples were taken upon incubation (T0; 2-3h after inoculation) as 663 well as after 24h (T1) and 48h (T2) of incubation. Asterisks represent significant differences with 664 control based on Wilcoxon Rank Sum tests with Holm correction ( $\alpha = 0.05$ ).

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Figure 5: Volcanoplots indicating gut microbial community alterations after 48h of in vitro batch incubations of fecal material from 10 donors with sugar depleted medium supplemented with 5 emulsifiers at 4 concentrations. Samples were taken upon incubation (T0; 2-3h after inoculation) as well as after 24h (T1) and 48h (T2) of incubation. Log2FoldChange (L2FC) of genus abundances for all emulsifier treatments versus the control are presented on the x-axis and the log transformed adjusted p-value is presented on the y-axis. Significantly increased or decreased genera are indicated in respectively green and red. The dashed line represents the significance threshold of  $\alpha = 0.05$ .

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Figure 6: Copy number corrected counts of significantly in- or decreased genera, obtained from DESeq analysis in R (version 3.4.2), after 48h of exposure of gut microbial communities from 10 donors to soy lecithin, sophorolipids and rhamnolipids during in vitro batch incubations. Asterisks represent significant differences with the control based on Wald tests ( $\alpha = 0.05$ ). bioRxiv preprint doi: https://doi.org/10.1101/2020.06.28.174946; this version posted June 28, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made **Dietary emulsifiers affect greentee of the second s** 



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Figure 7: Short chain fatty acid production levels over 48h of incubation of fecal material from 10 donors in sugar depleted medium supplemented with 5 emulsifiers at 4 concentrations. Samples were taken upon incubation (T0; 2-3h after inoculation) as well as after 24h (T1) and 48h (T2) of incubation.

684 Asterisks indicate significant differences with the control (0% (m/v)), calculated with Wilcoxon Rank

685 Sum tests with Holm correction. P-values indicate results of general Kruskal-Wallis tests ( $\alpha = 0.05$ ).

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Figure 8: Flagellin concentrations obtained using mTLR5 HEK blue reporter cells for three donors at the start (T0) and the end (T2) of in vitro 48h batch experiments of fecal material from 10 donors in sugar depleted medium supplemented with 5 emulsifiers at 4 concentrations. Donors for the flagellin assay were selected based on their low (D3), high (D7) and intermediate (D5) metabolic response to

692 the emulsifiers.

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Figure 9: Ranking of donors of fecal material for 48h batch incubations in sugar depleted medium supplemented with 5 emulsifiers at 4 concentrations. Donors were ranked along the principal parameters impacted by the emulsifiers. Measures were calculated based on cumulative (sum of all treatments) change in 48h production of SCFA or cummulative 48h change in living cell counts, relative or absolute abundances of *Escherichia/Shigella*.



700 **6 Tables** 

Table 1: Emulsifier characteristics of CMC, Span60, P80, phosphatidylcholine (major component of soy lecithin), sophorolipids and
 rhamnolipids.

	MW (g/mol)	CMC * (mg/L)	Source	Min. S.T. (mN/m)	Source
Carboxymethylcellulose	250000 1.2	-		-	
Sorbitan tristearate (Span60)	430.62 <sup>3</sup>	21.53	(Michor and Berg 2015)	62	(Parreidt et al. 2018)
P80	1310 <sup>4</sup>	0.012 mM =15.72 mg/L 0.033 mM =43.23 mg/L	(Pogorzelski, Watrobska-Swietlikowska, and Sznitowska 2012) (Bąk and Podgórska 2016)	+/- 43 36-38	(Bąk and Podgórska 2016) (Szymczyk, Zdziennicka, and Jańczuk 2018)
Phophatidylcholine	758.1 <sup>5</sup> 815.2 <sup>6</sup> 786.1 <sup>7</sup>	0.998 PC (16:0/16:0) 0.001 PC (18:0/18:0) 13 600 (lecithin)	(Zhang 2017) (Zhang 2017) (Wu and Wang 2003)	38 mN/m (PC) (21 mN/m for PI)	(Wu and Wang 2003) (Wu and Wang 2003)
Sophorolipids	706 8	150 27.17 10 20-130	(Kim et al. 2005) (Daverey and Pakshirajan 2010) (Otto et al. 1999) (Ma, Li, and Song 2012)	48 34.18 36 31-34.2 32.23-42.13	<ul> <li>(Kim et al. 2005)</li> <li>(Daverey and Pakshirajan 2010)</li> <li>(Otto et al. 1999)</li> <li>(Develter and Lauryssen 2010)</li> <li>(Ma, Li, and Song 2012)</li> </ul>
Rhamnolipids	650.79°	50-500 18.75 15-20 30	(Li et al. 2019) (Moussa, Mohamed, and Samak 2014) (Hörmann et al. 2010) (Vu, Tawfiq, and Chen 2015)	28 34 25-30 29.4 30-35	(Li et al. 2019) (Moussa, Mohamed, and Samak 2014) (Van Bogaert 2008) (Hörmann et al. 2010) (Vu, Tawfiq, and Chen 2015)

703 \*critical micelle concentration

## Dietary emulsifiers affect gut microbiota

- 704 \*\*minimal surface tension in aqueous solution
- 705 <sup>1</sup> https://www.sigmaaldrich.com/catalog/product/aldrich/419311?lang=en&region=CA (4-06-2020)
- 706 <sup>2</sup> https://pubchem.ncbi.nlm.nih.gov/compound/Carboxymethylcellulose-sodium (4-06-2020)
- 707 <sup>3</sup> https://www.sigmaaldrich.com/catalog/product/sial/85546?lang=en&region=CA (4-06-2020)
- 708 <sup>4</sup> https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Product\_Information\_Sheet/p8074pis.pdf (4-06-2020)
- 709 <sup>5</sup> https://pubchem.ncbi.nlm.nih.gov/compound/Soybean-lecithin (23-01-2020)
- 710 <sup>6</sup> https://pubchem.ncbi.nlm.nih.gov/compound/Lecithins (23-01-2020)
- 711 <sup>7</sup> https://pubchem.ncbi.nlm.nih.gov/compound/6441487 (23-01-2020)
- 712 <sup>8</sup> https://pubchem.ncbi.nlm.nih.gov/compound/Sophorolipid (23-01-2020)
- 713 <sup>9</sup> https://pubchem.ncbi.nlm.nih.gov/compound/5458394 (23-01-2020)

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## 1026 8 Conflict of Interest

1027 The authors declare that the research was conducted in the absence of any commercial or financial 1028 relationships that could be construed as a potential conflict of interest.

#### 1029 **9** Author Contributions

1030 This research was performed under promotorship of Prof. Tom Van de Wiele, Prof. John Van Camp 1031 and Prof. Andreja Raikovic. Lisa Miclotte was responsible for execution of all experiments, data 1032 visualisation and statistical analysis, as well as the assembly and submission of the manuscript. Chris 1033 Callewaert assisted in the execution of the PICRUSt algorythm. DESeq analysis was performed in 1034 collaboration with Kim De Paepe. Library preparation and 16S rRNA gene amplicon sequencing was 1035 performed by Leen Rymenans, collaborator withing Jeroen Raes' group. All co-author have reviewed 1036 the manuscript before submission.

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#### 1043 12 Data Availability Statement

1044 The 16S rRNA Illumina MiSeq sequencing data generated for this study can be found in the NCBI-1045 database under accession number PJNR PRJNA63057