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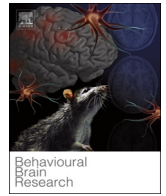
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## Research report

# First step of odorant detection in the olfactory epithelium and olfactory preferences differ according to the microbiota profile in mice

Laurent Naudon<sup>a,\*</sup>, Adrien François<sup>b</sup>, Mahendra Mariadassou<sup>c</sup>, Magali Monnoye<sup>d</sup>, Catherine Philippe<sup>d</sup>, Aurélia Bruneau<sup>d</sup>, Marie Dussauze<sup>b</sup>, Olivier Rué<sup>c</sup>, Sylvie Rabot<sup>d</sup>, Nicolas Meunier<sup>b</sup>

<sup>a</sup> Université Paris-Saclay, INRAE, AgroParisTech, CNRS, Micalis Institute, 78350, Jouy-en-Josas, France

<sup>b</sup> Université Paris-Saclay, UVSQ, INRAE, NBO, 78350, Jouy-en-Josas, F-78350, France

<sup>c</sup> Université Paris-Saclay, INRAE, MaIAGE, 78350, Jouy-en-Josas, France

<sup>d</sup> Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France

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## ABSTRACT

We have previously provided the first evidence that the microbiota modulates the physiology of the olfactory epithelium using germfree mice. The extent to which changes to the olfactory system depend on the microbiota is still unknown. In the present work, we explored if different microbiota would differentially impact olfaction. We therefore studied the olfactory function of three groups of mice of the same genetic background, whose parents had been conventionalized before mating with microbiota from three different mouse strains. Caecal short chain fatty acids profiles and 16S rRNA gene sequencing ascertained that gut microbiota differed between the three groups. We then used a behavioural test to measure the attractiveness of various odorants and observed that the three groups of mice differed in their attraction towards odorants. Their olfactory epithelium properties, including electrophysiological responses recorded by electro-olfactograms and expression of genes related to the olfactory transduction pathway, also showed several differences. Overall, our data demonstrate that differences in gut microbiota profiles are associated with differences in olfactory preferences and in olfactory epithelium functioning.

## 1. Introduction

Olfaction is a major sensory modality for most animal species. This sensory system is notably involved in food searching and food choice, in navigation, in environmental awareness and in recognition of conspecifics and social borders. Odour perception in terrestrial vertebrate species starts in the olfactory epithelium. It contains olfactory sensory neurons (OSN), which are renewed throughout the life of the animal [1]. OSNs detect odorants through cilia extruding into a mucus layer. These cilia contain the molecular component of the transduction pathway required to transform the chemical concentration information into an electrical signal that will be integrated into the brain [2]. We recently found that the olfactory system is altered in germfree mice [3]. Indeed, while the anatomy of the olfactory epithelium of germfree mice was not significantly different from conventionally reared conspecifics, their olfactory cilia layer was thinner and their cellular turn-over was

decreased. Furthermore, the amplitude of responses to odorants recorded electrophysiologically through electro-olfactograms (EOGs) was increased despite a lower level of transduction pathway component expression.

While our knowledge of the links between physiological functions and microbiota are growing constantly, notably thanks to the comparison between germfree animals and conventionally raised conspecifics, recent studies indicate that the nature of the microbiota plays a role in the observed effects on animal physiology. One of the most striking examples comes from the studies of metabolic disorders. For example, transplantation into mice of faecal microbiota from human twins discordant for obesity induces different metabolic phenotypes [4]. Similarly, transplantation of faecal microbiota from mice discordant for glucose metabolism induces different glycaemia levels and glucose tolerance patterns in the recipient mice [5,6].

In the present work, we wanted to explore to which extent different

*Abbreviations:* ACIII, adenylate cyclase III; Cnga2, cyclic nucleotide gated channel alpha 2; PDE, phosphodiesterase; EOG, electroolfactogram; Golf, olfactory-type G protein; OE, olfactory epithelium; OSN, olfactory sensitive neuron; SCFA, short chain fatty acids

\* Corresponding author.

E-mail address: [laurent.naudon@inrae.fr](mailto:laurent.naudon@inrae.fr) (L. Naudon).

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microbiota could relate with differences in olfaction. We conventionalized germfree mice of a given genetic background with microbiota from three different mouse strains. The C3H/HeN inbred strain was chosen as the germfree recipient strain and the C3H/HeN, BALB/cByJRj and RjOrl:Swiss mice as the conventional donors, since previous studies have shown marked differences in the composition of their microbiota [7–9]. Conventionalized mice were mated, and all the experiments were performed on the adult male offspring. This procedure allowed us to study mice that had been exposed to microbiota early in life and whose brain could thus develop normally [10]. Differences in gut microbiota composition between the 3 groups were ascertained using amplicon sequencing of the 16S rRNA marker gene (meta-barcoding). The olfactory system was investigated using behavioural, electrophysiological and molecular approaches.

## 2. Material and methods

### 2.1. Animals

Procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals and approved by the local Ethics Committee (approval reference: 14–42). Animal experiments were carried out in the Anaxem germfree facility (Micalis Institute, INRA, Jouy-en-Josas, France). Five-week-old male and female conventional specific pathogen free (SPF) mice of the C3H/HeN, Swiss and BALB/cByJ strains were purchased from Janvier Labs (Le Genest Saint Isle, France). Three-week-old male and female germfree mice of the C3H/HeN strain were obtained from the Anaxem germfree breeding facility. The conventional mice were placed in sterile isolators (one isolator/mouse strain); males and females were housed in separate cages. After 1 week of acclimatization, 3-week-old C3H/HeN germfree males and females were co-housed for two weeks with the conventional males and females, respectively. As mice are coprophagic and perform many social interactions, this co-housing would allow the colonization of germ free mice by the conventional mice microbiota. At the end of this period, the conventional mice were removed and the conventionalized ex-germfree mice were mated at the age of 6 weeks (six breeding pairs per isolator). After the birth, the litters were left undisturbed in the presence of both parents until weaning. At that time, the entire male offspring, which consisted in 18 mice in each isolator, was housed in collective cages until adulthood. The groups of C3H/HeN mice harbouring the microbiota of C3H/HeN, Swiss or BALB/cByJ mice, were named C3H-C3H, C3H-Swiss and C3H-BALB/c, respectively. At 8–10 weeks of age, 13–15 mice per group were used for a spontaneous odour preference test. Among the mice having performed the behavioural test, 8–10 mice per group were chosen randomly and used for electro-olfactogram (EOG) and qPCR analysis as both techniques require only one hemi head to be performed. The faeces from the 5 remaining mice plus 3–5 mice that did not perform the behavioural tests were used for the 16S rRNA amplicon sequencing. Mice were always kept in standard cages containing sterile bedding made of wood shavings, and given free access to autoclaved tap water and a  $\gamma$ -irradiated (45 kGy) standard diet (R03; Scientific Animal Food and Engineering, Augy, France). The animal room was maintained at 20–24 °C and kept on a 12 h light/dark cycle (lights on at 7:30 am). The mice were euthanized by decapitation following sodium pentobarbital anaesthesia.

### 2.2. Metagenomic DNA extraction, 16S rRNA amplicon sequencing and bioinformatic analysis

The bacterial DNA was extracted from mice faeces using Genome DNA Isolation Kit (MP Biomedicals, Santa Ana, CA, USA). The V3-V4 region of the 16S rRNA genes was amplified using KAPA2G Robust PCR kit (KAPABiosystems) and the primers V3F: CTTCCCTACACGACGCTCTCCGATCTACGGGAGGCAGCAG and V4R: GGAGTTACAGACGTGT

GCTCTCCGATCTTACCAGGGTATCTAATCCT. Purified amplicons were sequenced using the MiSeq sequencing technology (Illumina, San Diego, CA, USA) at the GeT-PLaGe platform (Genotoul, Toulouse, France). Paired-end reads obtained from MiSeq sequencing were analyzed using the Galaxy supported pipeline named Find RapidlyOperational Taxonomic Units (OTUs) with Galaxy Solution (FROGS) (33). For the preprocessing, only reads with length  $\geq 380$  bp were kept. The clustering and chimera removal steps followed the guidelines of FROGS (Escudié et al. 2018). Taxonomic assignment was performed using the Silva database (v123, <https://www.arb-silva.de/>). OTUs with global abundance lower than 0.005 % were removed from the analysis following (Bokulich et al. 2013).

### 2.3. Odorants

Odour stimuli comprised a battery of eleven odorants (Sigma Aldrich, Saint-Quentin Fallavier, France, except for 2,5-dihydro-2,4,5-Trimethylthiazoline (TMT), Contech, Vancouver, Canada) and a rat faeces extract. This last extract was obtained after mixing 5 g of fresh adult male rat faeces with 5 mL of mineral oil in a sealed vial, letting this mixture marinate at 65 °C for 1 week, then keeping the supernatant. Odorants were diluted in mineral oil. The odorants were chosen for their potentially varied attractiveness to mice.

### 2.4. Spontaneous odour preference test

The spontaneous preferences of 8–10-week-old mice for various odorants were determined by measuring the amount of time spent investigating each odorant with experimenters blind to the identity of the odorant. All behaviour experiments were conducted in isolators to stabilize the microbiota of each mouse group and we used a simple test that was effective in a previous study in rats [11]. During a first session, individual mice were presented with 6 different odorants one at a time, as well as mineral oil twice as a control. The second session took place one week later with 6 new odorants and twice the mineral oil. The order in which odorants were presented each testing day was randomized across mice, but the two presentations of the mineral oil were always in the third and sixth positions. The odorants were stored in glass vials sealed with a plastic cap that allows samples being drawn with a syringe. To present the odours during the tests, 1 mL syringes with their ends cut off (length 6.5 cm, diameter 7 mm) were used. A square of Whatman filter paper (2 cm x 2 cm) accordion-folded 4 times was placed in the tip and impregnated with 500  $\mu$ L of an odorant, then at each presentation a drop of this odorant was applied to the paper. The testing took place in the isolator, in a standard clean rat cage (LxWxH: 36 cm x 21 cm x 19 cm) covered with a metal grid. The cage floor was devoid of bedding. Each odorant vial and syringe were individually enclosed in a sealed tube to limit the spread of odours in the isolator. At the beginning of a testing session, a mouse was placed at one end of the testing cage during 2 min in the presence of an empty syringe positioned at the opposite end, in the middle of the metal grid 3 cm from the wall. The empty syringe was replaced by a syringe with a piece of paper impregnated with the first odorant. The duration of active investigation, defined as time during which the nose of the mouse was within 1 cm of the piece of paper, was measured for a period of 3 min. The syringe was then removed from the metal grid, and the mouse was left in the cage with no syringe present for 2 min before presentation of the next odorant. The syringe was cleaned with a paper towel made damp with water before being enclosed in a seal tube. After the last odorant, the mouse was put back in its home cage. The pellets and urine were removed from the cage floor using another damp paper towel. Finally the test cage was dried before placing the next mouse. Each session was videotaped then scored manually by an observer blind to the treatment. To analyse the data, we first normalized investigation times for each mouse individually by dividing the duration of investigation for each specific odorant by the average investigation time

of that mouse in that session. Then, the normalized investigation duration for the mineral oil was subtracted from the normalized investigation duration of each specific odorant. Data are expressed as medians (interquartile range).

### 2.5. Electro-olfactogram recording (EOG) of the olfactory epithelium

To compare the global responses of OSN from the 3 groups of mice, EOG recordings were made from the OE in an opened nasal cavity configuration as described earlier [12]. Mice were euthanized during the light phase 09:00-18:00 to allow continuous recordings during the working day. We took care to alternate them according to their group to limit any circadian bias. The hemi-head was placed in a recording chamber under an upright Olympus SZ51 stereo microscope Olympus, Rungis, France equipped with a low magnification objective 0.8 to 4x and two MX-160 micromanipulators Siskiyou, Inc., Grants Pass, OR, USA. The hemi-head was kept under a constant flow of humidified air filtered through activated charcoal ~1000 ml/min delivered through a 9 mm glass tube. This tube was positioned 2 cm from the epithelial surface. Odour stimulations were performed by puffs of air 200 ms, 200 ml/min blown through an exchangeable Pasteur pipette enclosed in the glass tube containing a filter paper impregnated with 20 µL of odorant. We used the following dilution in mineral oil of odorants volume/volume: heptanal ranging from 1:10,000 to 1:10; S-methyl thiobutanoate SMB, acetophenone, propionic acid and 2,5-dihydro-2,4,5-Tri-methylthiazoline TMT at 1:10,000; - carvone, eugenol, cadaverine and phenylethylamine at 1:100; heptanoic acid and rat faeces extract at 1:10. We chose these odorant dilutions based on their ability to trigger an average response in EOG around 10 mV, except for carvone which was used as a positive control for the quality of the preparation and applied at the beginning and at the end of all experiments and for some compounds where higher concentrations would impair the subsequent responses in EOG cadaverine, propionic acid, phenylethylamine. Voltage signals were recorded using an XtraCell 2 channels amplifier DIPSI, Châtillon, France used in a DC current-clamp configuration  $I = 0$ , low-pass Bessel filtered at 1 kHz and digitized at a rate of 2 kHz using a Digidata 1322a A/D converter Axon Instruments, Molecular Devices, Union City, CA, USA interfaced to a Pentium PC and Pclamp 9.2 software Axon Instruments. A reference Ag/AgCl electrode was placed on the frontal bone overlaying the olfactory bulb. Recordings were made with glass micropipettes of 4-5MΩ filled with a saline solution. EOG were recorded from the centre of turbinates IIB and III (Fig. 3A). These positions gave robust, reproducible and long-lasting EOG recordings ranging from 12 to 20 mV when stimulated with (-) carvone 1:100. Odorant-free air stimulation (with mineral oil) always produced signals around 1 mV amplitude. Analyses were performed using Clampfit 9.2 (Axon Instruments) to measure peak amplitude. Data are expressed as mean ± standard error of the mean (SEM).

### 2.6. qPCR gene expression analysis in the olfactory epithelium

Total RNA was extracted from frozen olfactory mucosa using the Trizol method. OligodT first strand cDNA were synthesized from 5 µg of previously DNase I treated total RNA by the Superscript II reverse transcriptase (Invitrogen) following the manufacturer's recommendations. For quantitative PCR, 5 µl of 125-fold diluted cDNA templates were added to the 15µl-reaction mixture containing 300 nM primers (sequences in) and SYBR Green GoTaq® qPCR Master Mix (Promega, Charbonnières, France). The PCR was performed on a Mastercycler® ep realplex (Eppendorf) for 40 amplification cycles consisting of 45 s at 94 °C, 45 s at 60 °C and 45 s at 72 °C. Quantification was achieved using the  $\Delta\Delta C_t$  method. mRNA expression was normalized to the expression level of either the OSN specific  $\beta$ -tubulin III for OSN expressed genes or  $\beta$ -actin for genes related to perireceptors events (OBPs and detoxifying enzymes), which are also expressed by non-neuronal cells of the olfactory mucosa [12]. An efficiency corrective factor was applied for

each primer pair.

### 2.7. Short-chain fatty acid analysis in caecal contents

SCFA analysis was carried out as described previously [13]. Caecal contents were collected and stored at -80 °C. Thawed samples were water extracted and proteins were precipitated with phosphotungstic acid. A volume of 0.1 µL supernatant fraction was analysed for SCFA on a gas-liquid chromatograph (Autosystem XL; Perkin Elmer, Saint-Quentin-en-Yvelines, France) equipped with a split-splitless injector, a flame-ionisation detector and a capillary column (15 m x 0.53 mm, 0.5 µm) impregnated with SP 1000 (FSCAP Nukol; Supelco, Saint-Quentin-Fallavier, France). Carrier gas (H<sub>2</sub>) flow rate was 10 mL/min and inlet, column and detector temperatures were 200 °C, 100 °C and 240 °C, respectively. 2-Ethylbutyrate was used as the internal standard. Samples were analysed in duplicate. Data were collected and peaks integrated using the Turbochrom v 6 software (Perkin Elmer, Courta-boeuf, France).

### 2.8. Statistics

16S rRNA amplicon analysis. The olfactory preference test and the SCFA data were analysed using one-way ANOVA followed by Tukey's HSD post-hoc test. EOG and gene expression data were analysed with a one-way ANOVA followed by Bonferroni multiple comparison post-hoc tests except for the EOG dose response to heptanal stimulation which was analysed with a two-way ANOVA. A probability value of  $P < 0.05$  was used as an indication of significant differences. Calculations were performed using GraphPad Prism software (version 5.04, La Jolla, CA, USA).

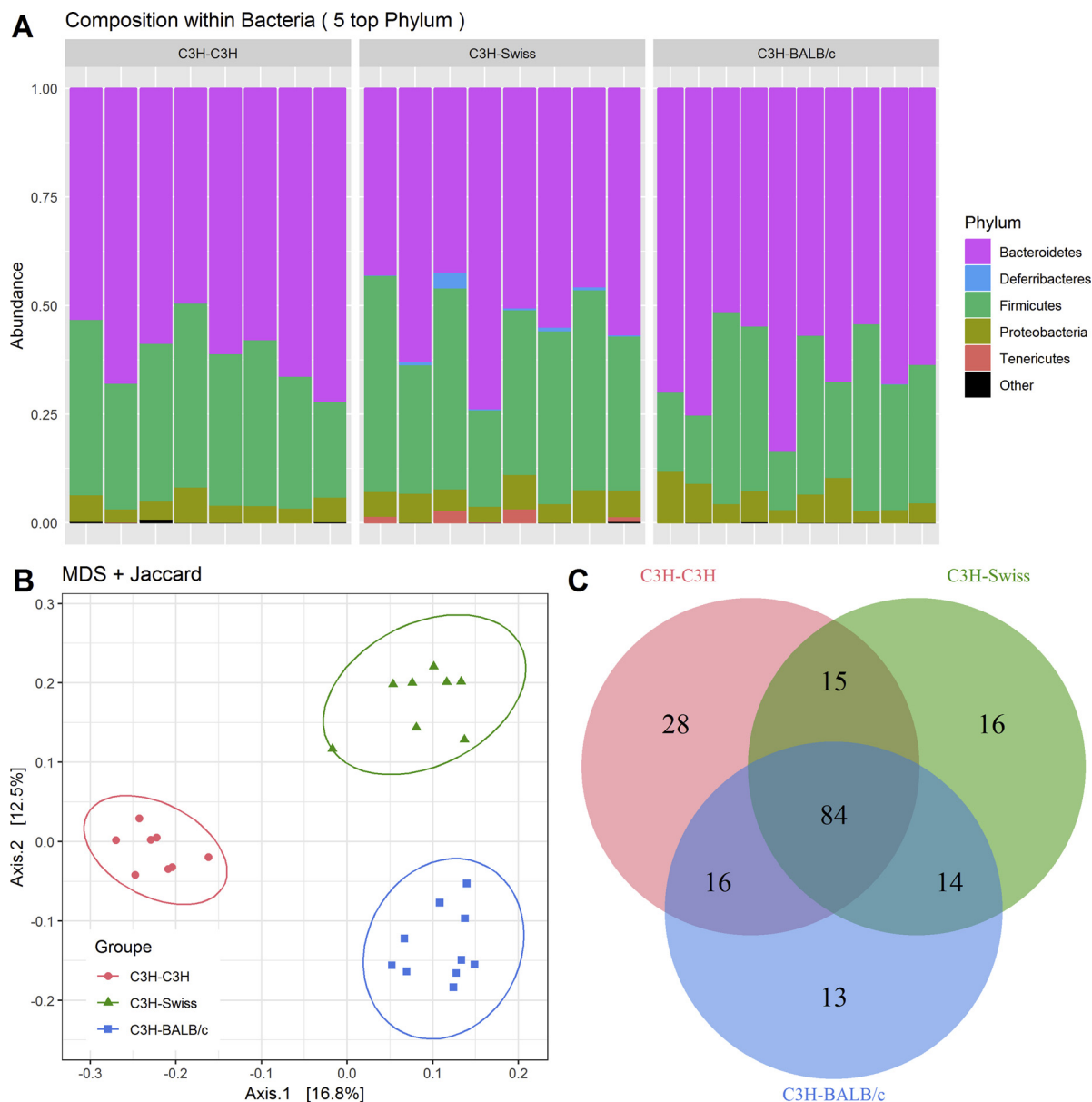
## 3. Results

### 3.1. Analysis of the 16S rRNA gene sequences indicated that the gut microbiota differed in terms of species repertoire between C3H-C3H, C3H-Swiss and C3H-BALB/c mice

The samples were rarefied to the same sample depth (1626 reads per sample) prior to all diversity analyses. The  $\alpha$ -diversity (measured by the Shannon index) was similar between the three groups (One way ANOVA followed by Tukey's HSD post-hoc test,  $P > 0.05$ ), indicating similar diversity levels in the 3 groups. Likewise, phylum level composition profiles were similar in the three groups (Fig. 1A). Beta diversities between all samples were computed on all samples using the Jaccard distance and the resulting pairwise distance matrix was used to perform a principal coordinate analysis (PcoA also known as MDS). The PCoA plot shows that samples cluster by group, and that the groups are well separated (Permutational multivariate ANOVA with adonis,  $P < 0.001$ ). This separation disappears when considering the Bray-Curtis distance (not shown) (Fig. 1B). This was reflected by the large number of group specific OTUs, as shown in the Venn diagram (Fig. 1C). Therefore, although the groups may share their abundant taxa (and thus have low Bray-Curtis distances), each one has a long tail of group-specific subdominant taxa (as evidenced by high Jaccard distances).

### 3.2. Different gut microbiota profiles are associated with different olfactory preferences

As microbiota differences occurred between the C3H-C3H, C3H-Swiss and C3H-BALB/c mice, we next examined whether those differences could be associated to changes in the attractiveness of various odorants for these mice. We measured the time they spent investigating 12 different odorants during a 3 min assay (Fig. 2A). Those tests were performed in two sessions separated by one week with the assay of 6 odorants and mineral oil alone in each session. We assessed



**Fig. 1. Gut microbiota composition differed in C3H mice harbouring microbiota from different mouse strains.** Analysis was based on 16S rRNA gene sequencing. (A) Bar graph shows the relative abundance of major phyla in each sample. (B) PCoA ordination plot of the Jaccard distance matrix between samples. The microbiota differ significantly (permutational multivariate analysis of variance with adonis,  $P < 0.001$ ) between the 3 groups of C3H/HeN mice harbouring microbiota from conventional C3H/HeN (C3H-C3H), Swiss (C3H-Swiss) or BALB/cByJ (C3H-BALB/c) mice ( $n = 8-10$ ). (C) Venn diagram representing shared and specific OTUs between the 3 groups. Although there is a large group of “core” OTUs shared across the 3 groups, each group has many group-specific OTUs.

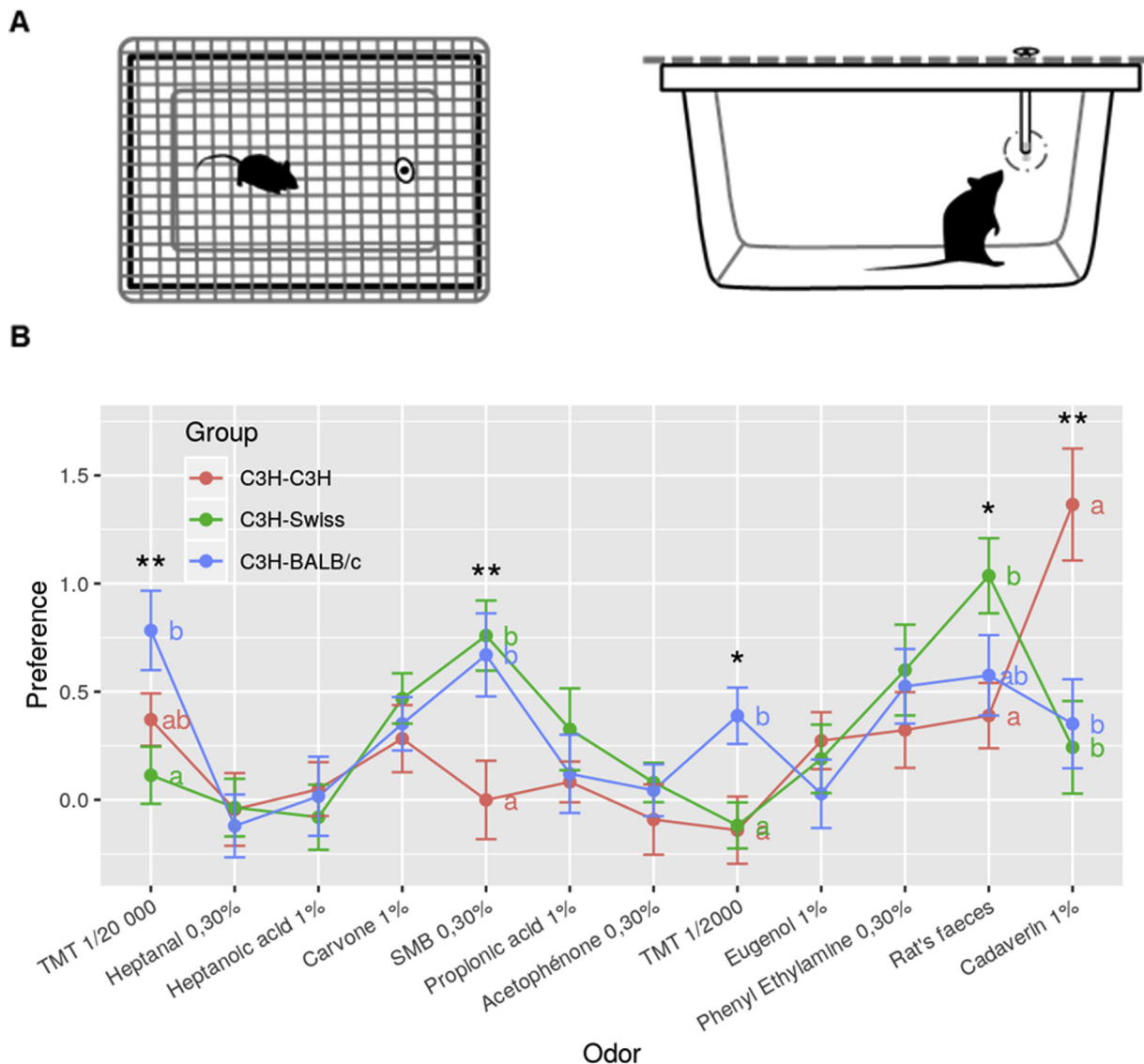
the average spontaneous investigation time during the two sessions for all odorants. It was similar among the 3 groups (C3H-C3H,  $2.48 \pm 0.2$  s; C3H-Swiss,  $2.32 \pm 0.18$  s; C3H-BALB/c,  $2.37 \pm 0.19$  s; mean value  $\pm$  SEM,  $n = 13-15$ ,  $P = 0.82$ ). These values of spontaneous investigation time were consistent with previous studies [14].

The data obtained from normalized investigation time (Fig. 2B) showed that mice had a neutral attitude to some odorants (values close to zero) or a tendency to prefer some odorants (values tending to 1, or exceeding it). It may be noted that, in our conditions, none of the odorants caused a statistically significant aversive reaction in the mice (data not shown). According to the bacterial status, one-way anova followed by Tukey’s HSD post-hoc tests evidenced differences in preferences for some of the odorants. This was the case for cadaverine at 1% ( $P = 0.0017$ ), S-methyl thiobutanoate (SMB) at 0.3% ( $P = 0.0071$ ), TMT at 1/20,000 ( $P = 0.034$ ) and 1/2,000 ( $P = 0.0095$ ) dilutions, and for the rat faeces extract ( $P = 0.026$ ). The C3H-BALB/c mice exhibited

a significantly higher preference for TMT than C3H-Swiss mice at both concentrations tested, whereas this preference was only significant at the lower concentration when compared to that of C3H-C3H. In contrast, the C3H-C3H displayed a higher preference for cadaverine at 1% and a lower preference for the SMB at 0.3% than both C3H-Swiss and C3H-BALB/c mice. Finally, C3H-Swiss mice presented a significantly higher preference for the rat faeces extract than C3H-C3H mice.

### 3.3. Different gut microbiota profiles are associated with changes in olfactory epithelium odorant detection

To assess whether the microbiota differences could be associated with changes in the OSN responses to odorants, we performed EOG recordings in mice that had been subjected to the olfactory behavioural test ( $n = 8$ ). We stimulated the OE with increasing concentrations of heptanal as well as several other odorants used in the behavioural test

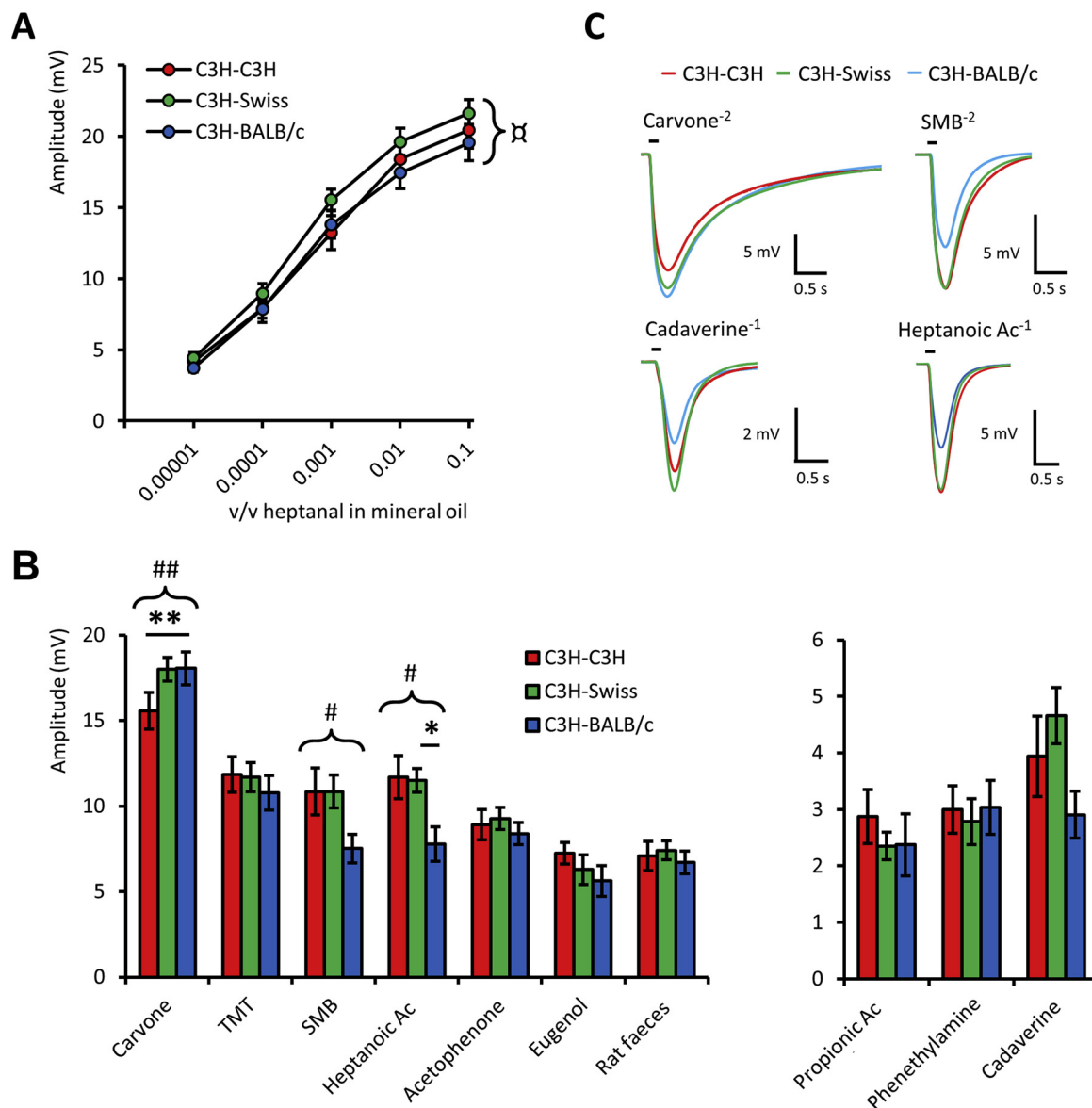


**Fig. 2. Spontaneous investigation times of odorants differ according to the microbiota status of mice.** (A) Schematic diagram of the experimental set-up seen from above and from the side; active investigation was defined as the nose of the mouse being within 1 cm of the tip of the syringe (area represented by the stippled circle) containing a piece of odour-soaked filter paper. (B) During two sessions, conducted a week apart, individual mice were presented with 6 different odorants one at a time, as well as mineral oil twice as a control. The results are presented as mean of the normalized investigation time for each odorant, subtracted from the normalized investigation time for mineral oil (referred to as preference in the graph) for C3H-C3H, C3H-Swiss or C3H-BALB/c mice ( $n=15, 15, 13$ ). Results are expressed as mean  $\pm$  SEM. For each odour, a one-way ANOVA was performed to identify odours that discriminate the groups (\* $P < 0.05$ ; \*\* $P < 0.01$ ). Groups with different superscript letters (a, b) are significantly different (Tukey's HSD post-hoc test,  $p < 0.05$ ).

(Fig. 3A). We measured the maximum amplitude of responses to these odorants. Two-way ANOVA performed on the dose response to heptanal showed that curves were different according to the mice bacterial status ( $F_{(2,215)} = 3.616$ ;  $p = 0.028$ ) (Fig. 3B). Furthermore, several other odorants induced different responses (Fig. 3C–D). One-way ANOVA indicated that the peak amplitudes were different among the three groups for the stimulation with carvone ( $P = 0.002$ ), SMB ( $P = 0.042$ ) and heptanoic acid ( $P = 0.017$ ). Post Hoc Bonferroni test indicated that the response to carvone was lower in the C3H-C3H group compared to the C3H-BALB/c and that the response to heptanoic acid was higher in the C3H-Swiss group compared to the C3H-BALB/c group. Interestingly, while the behavioural interest of the mice towards cadaverine, TMT and rat faeces differed among groups, we did not observe significant differences among those groups in EOG recordings when stimulated with these odours.

#### 3.4. Changes in expression of genes related to odorant detection in the OE are associated with differences in the gut microbiota

As the EOG recordings were different depending on the mice group, we analysed the expression level of various genes related to the olfactory signal transduction and odorant degradation ( $n = 8$ ). Most volatile odorants are transduced through the activation of an olfactory receptor coupled to a G protein ( $G_{olf}$ ), which in turn activates the adenylate cyclase III (ACIII). The rise in cAMP level then activates a CNG channel (Cnga2) and this rise is counterbalanced by the cAMP degrading phosphodiesterase PDE1C2. One-way ANOVA showed that with the exception of  $G_{olf}$  expression ( $P = 0.4$ ), all genes related to the olfactory transduction cascade were significantly expressed at a higher level in C3H-C3H mice compared to the two other mice groups (Fig. 4A,  $P = 0.017$  for ACIII,  $P = 0.0095$  for Cnga2,  $P = 0.0085$  for PDE1C2). We did not observe any variation in the three olfactory receptor gene expressions we tested (Data not shown for Olfr151, 2 and 937 expression

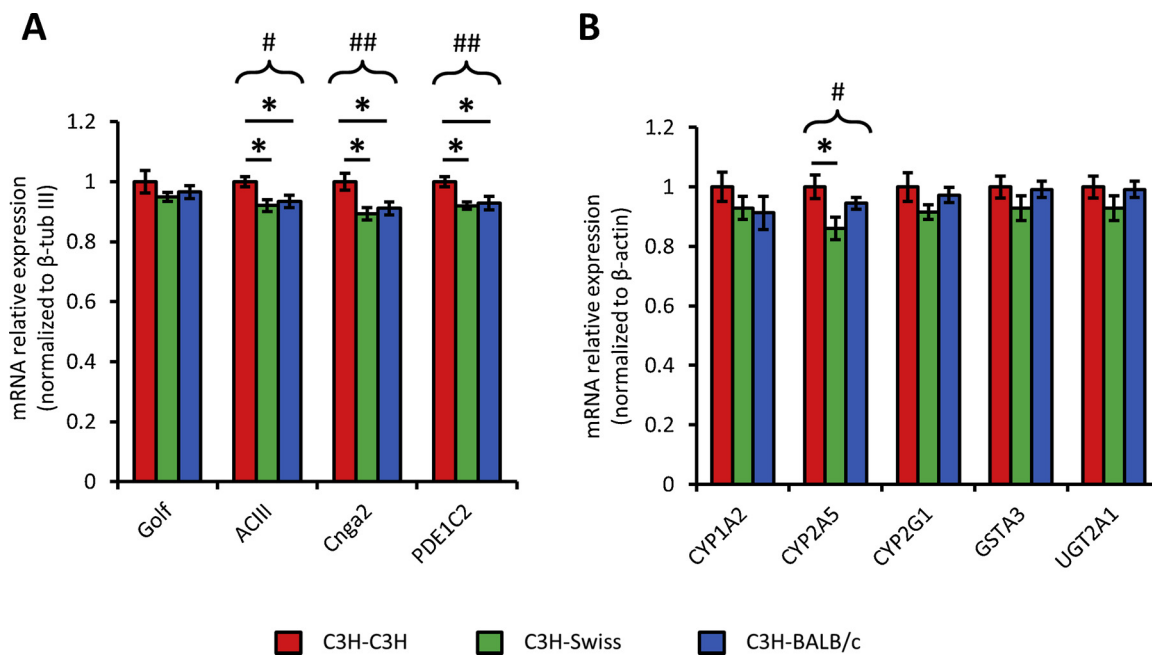


**Fig. 3. Olfactory epithelium responses to odorants differ according to the microbiota status of mice.** The global response of the olfactory sensory neurons from the 3 groups of mice was assessed by electro-olfactogram. (A) Schematic diagram of hemi head experimental preparation. Responses from the olfactory epithelium of turbinates IIB and III were recorded during stimulation with (B) increasing concentrations of heptanal and (C) various odorants. Values represent the mean of peak amplitude  $\pm$  SEM ( $n = 8-10$ ), two-way ANOVA for dose response curve to heptanal  $^{\#}P < 0.05$ ; one-way ANOVA among the three groups for other odorants,  $\#P < 0.05$ ;  $\#\#P < 0.01$  followed by Bonferroni post-hoc test  $*P < 0.05$ ;  $**P < 0.01$ . (D) Average traces for four of the odorants tested. Small black line on top of recordings indicates odorant stimulation.

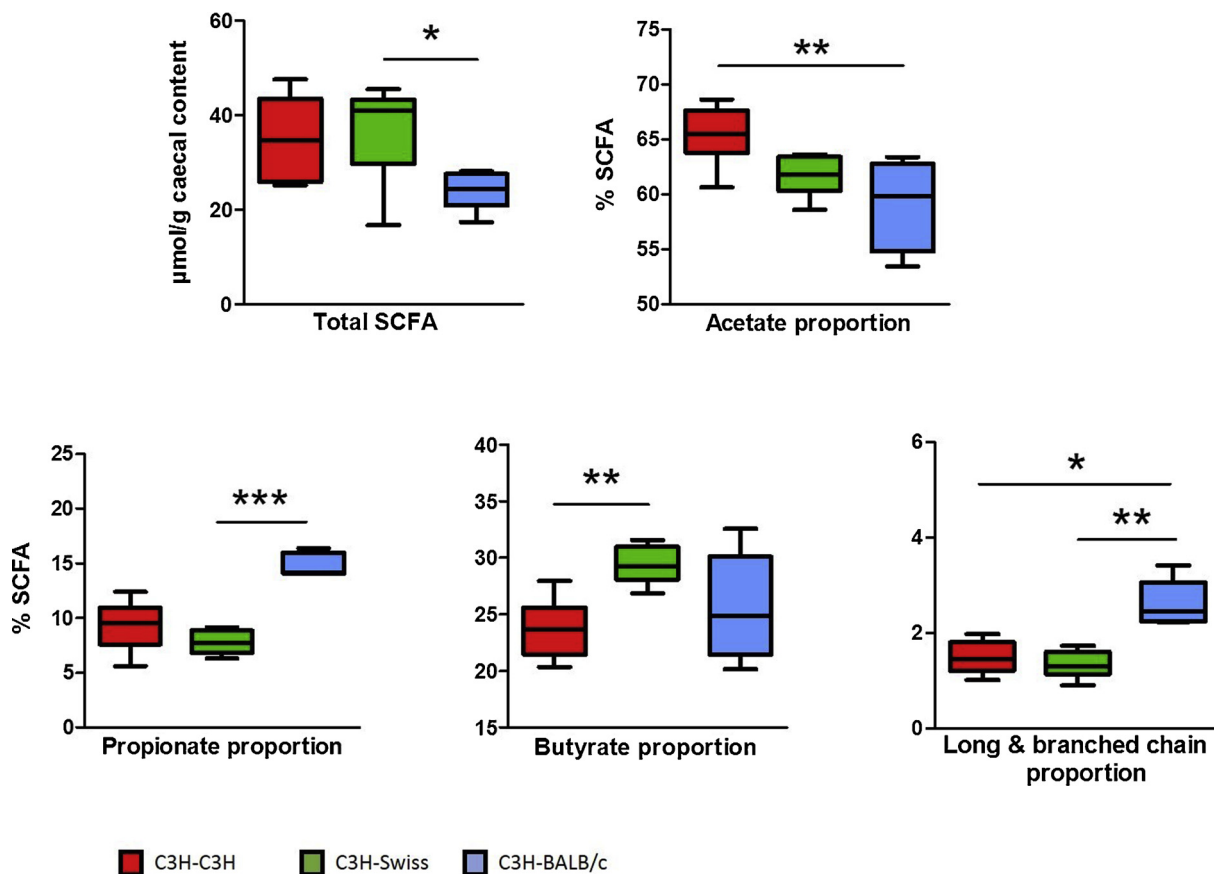
levels known to be activated by acetophenone, heptanal and heptanoic acid, respectively). Odorants are rapidly eliminated in the OE through the activity of various enzymes, involving mainly cytochrome P450 enzymes, glutathione-S-transferase (GST) and UDP-glucuronosyl transferase (UGT). We examined the expression of the genes encoding the main machinery of degradation enzymes in the OE [15]. The expression level was not statistically different for most of these with the exception of the enzyme CYP2A5 expressed at a higher level in C3H-C3H mice compared to C3H-Swiss group (Fig. 4B,  $P = 0.027$ ). Odorants are hydrophobic and olfactory binding proteins are thought to improve the solubilisation of odorants in the nasal mucus, allowing them to reach the olfactory receptors [15]. We also explored whether the expression of the olfactory binding proteins was altered by the mice bacterial status but did not find any significant differences (data not shown).

### 3.5. The 3 mice groups have different caecal short-chain fatty acid profiles

We analysed the caecal SCFA profiles of 5-7 mice per group as a biomarker of change of mice body odours (Ezenwa and Williams, 2014). The caecal SCFA overall concentration was significantly lower in the C3H-BALB/c mice compared with the C3H-Swiss mice (Fig. 5,  $P = 0.041$ ). C3H-BALB/c SCFA profiles were also characterized by a significantly greater proportion of long- and branched-chain fatty acids, which mainly arise from protein fermentation, compared with the two other groups ( $P = 0.003$ ). In those mice, we also observed a significantly greater proportion of propionate compared with the C3H-Swiss group ( $P = 0.0015$ ), and a significantly lower proportion of acetate compared with the C3H-C3H group ( $P = 0.0095$ ). C3H-C3H and C3H-Swiss mice differed significantly by a greater proportion of butyrate in the latter group ( $P = 0.0094$ ). Overall, these results show that the microbiota metabolic profiles differed among the 3 groups of



**Fig. 4. Modulation of the expression of genes related to odorant detection in mice with different microbiota.** Quantitative PCR analysis of cDNAs from olfactory mucosa. (A) Main olfactory transduction pathway components; (B) detoxifying enzymes. Their expression levels were normalized to that of  $\beta$ -tubIII (A) or  $\beta$ -actin (B) and are given as mean  $\pm$  SEM (n = 8–10). One-way ANOVA among the three groups, #*P* < 0.05; ##*P* < 0.01 followed by Bonferroni post-hoc test \**P* < 0.05.



**Fig. 5. Caecal SCFA profiles differ in C3H mice harbouring microbiota from different mouse strains.** Caecal concentration and relative proportions of short chain fatty acids (SCFA) in C3H/HeN mice harbouring microbiota from conventional C3H/HeN (C3H-C3H), Swiss (C3H-Swiss) or BALB/cByJ (C3H-BALB/c) mice (n = 7, 7, 5). The median is represented by a bar inside a box. The box indicates the interquartile range, the chart goes down to the smallest value and up to the largest, Kruskal-Wallis test followed by Dunn's post-hoc pairwise comparisons \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



mice. As those were all fed on the same diet, this reflected different gut microbiota profiles leading to different body odour profiles.

#### 4. Discussion

A growing amount of data indicates that the microbiota influences many physiological parameters in mammals [16]. This has been mainly shown by comparing germfree *versus* conventional animals. As such, we observed that the absence of microbiota modulates the first step of the olfactory system. We found that while no profound changes occurred in the anatomy of the olfactory epithelium, the cellular renewal was decreased in germfree animals while responses to odorants were increased [3]. In the present study, we asked whether characteristics of the olfactory system would change according to microbiota variations.

Genetic analysis techniques, particularly the 16S rRNA amplicon sequencing revealed variations in microbiota among mouse strains and showed that the difference between microbiota profiles was higher for genetically distant mice strains, indicating that the microbiota composition was affected by the genetic of the host [8]. We thus colonized germfree C3H/HeN mice with the microbiota from the same strain or from genetically distant strains, namely BALB/c and Swiss. Furthermore, we chose to study the offspring of these ex-germfree C3H/HeN mice as they hold a microbiota during their whole development. We first confirmed by an analysis of the 16S rRNA gene sequences that the microbiota of the 3 groups of F1 generation mice, named C3H-C3H, C3H-Swiss and C3H-BALB/c, were indeed different. We could thus examine if these mice, with the same genetic background but different microbiota, would differ in their olfactory system.

We first examined if olfactory preferences were different among the three groups of mice. We designed a simple test manageable in a sterile isolator, based on a previous study [11]. We used various odorants known to be either attractive or repulsive in mice [17] and even produced a home-made extract from rat faeces as rat odour is avoided by mice [18]. We limited the study to odorants known to impact olfactory behaviour and volatile enough to induce significant signals in EOG. We chose odorant concentrations matching average signals elicited in electrophysiological recordings in order to be able to compare potential differences. Surprisingly, whatever the odorant used, we did not observe any significant decrease of the odorant investigation time compared to mineral oil used as a control. Some odorants such as TMT [17] and phenylethylamine [19] are known to induce innate fear in mice that can be revealed by the time the animal stays immobile whilst alert (referred commonly as freezing). We thus also monitored the episodes of freezing during the behaviour test but did not observe any. For TMT, we even observed an increase in mice interest reflected in increased odorant investigation time. Such observations have also been recently reported by others [20] and TMT appears to be repulsive to mice only at high concentrations. Overall, among the eleven odorants evaluated (TMT was tested twice with different concentrations), mice responded differently to four of them. Furthermore the difference in investigation times was dependent on the group of mice. For instance, the C3H-C3H mice group was more interested in cadaverine than the two other groups, while TMT was more attractive to the C3H-BALB/c mice. Thus, the variation in olfactory preferences observed cannot be assigned solely to a global alteration of the olfactory system as they depend on the identity of the odorant and differ according to the microbiota profile of the mouse. While this is to our knowledge the first study showing an association between microbiota variations and a sensory system, this result is consistent with the growing data showing a relationship between the nervous system and the microbiota.

We then explored electrophysiologically if the behaviourally tested odorants elicited different signals in the olfactory epithelium. Again, several odorants differentially activated the olfactory epithelium according to the microbiota profile, confirming the importance of the microbiota in the olfactory epithelium physiology. The finding that odorants elicit different EOG patterns according to the microbiota

profile points to an additional specificity of the microbiota influence depending on the odorant identity. We also observed moderate but significant differences in the transduction pathway genes expression as well as one gene coding for an enzyme related to odorant clearance. Globally the expression of those genes was slightly higher in the C3H-C3H mice indicating again the importance of microbiota profile in olfactory epithelium physiology. However, such slight change cannot be directly linked to the impact of microbiota on EOG responses.

How could the microbiota be related to the detection of odorants at the level of the olfactory epithelium? A growing number of studies indicates that the olfactory epithelium properties are dependent on the presence of environmental odorants [21–24] and it has been clearly established that the microbiota produces an important amount of odorants relevant for animal olfactory related behaviour [25,26]. Our caecal SCFA analysis clearly show that the different groups of mice are producing different odours in their faeces. The odorant signature of the microbiota in which SCFAs play a major role due to their abundance and volatile nature could thus indirectly affect the properties of the olfactory epithelium. A recent study in human linking olfactory performances with microbial community composition and particularly butyrate producing micro-organisms with poor olfactory performances is consistent with this hypothesis [27].

We used the same odorants in EOG recordings and in olfactory behavioral test. However, the variation in olfactory preferences and EOG signals were not correlated. Indeed, SMB was the only odorant eliciting inter-group differences with both experimental approaches, with variations depending on the group: while the C3H-C3H group was the least interested in SMB, it was in the C3H-BALB/c group that SMB elicited the smallest EOG signal. Olfactory behaviour is the result of the integration of odorant detection in the olfactory mucosa by the central nervous system. The discrepancy between the changes at the level of the olfactory epithelium and in olfactory preferences suggests that both the olfactory epithelium properties and the central processing of odorant hedonic value are likely influenced by the microbiota. How could the microbiota affect the hedonic value of odorants? In the same way that environmental odorants have impact at the olfactory epithelium level [24], the central processing of odorants is also conditioned by early animal olfactory experiences [28,29]. In our study, different odorant combinations produced by the gut microbiota such as the short-chain fatty acids could thus have affected the hedonic value of the odorants used in the behavioral test. Accordingly, the extent to which a mouse explores a novel odour vary greatly, depending on the strain [30]. Naturally, further studies are required to evaluate the importance of the microbiota in the variation in olfactory related behaviour among mice strains. Interestingly, social discriminations in mice are modulated by early olfactory experience [31], thus the microbiota could influence social interactions by participating in the olfactory signature of an animal. This could partly explain recent results indicating that divergence in the composition of the gut microbiota in mice of the same genetic background may be accompanied by differences in social interaction behavior [32]. Recently, the performance of germfree mice, and germfree mice inoculated with a microbiota from mice or guinea pigs were assessed in a preference test to male and female odors. A preference for male odor by female mice was only observed in the germfree mice inoculated with mice microbiota. This result reinforces the idea that the microbiota during the juvenile period is important for the development of normal odor preference [33]. Olfactory preferences are also essential in feeding behaviour [34] and the diversity of food ingested has an impact on the microbiota [33,35]. Our present work indicates that the microbiota could play a key role in shaping the chemical senses and thus the feeding behaviour of mammals as it has recently been shown in insects [36–39]. It could be one piece of the puzzle in the microbiota-brain axis, as stabilizing the type of food ingested will in turn maintain the microbiota population.

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## Author contributions

AF, LN, NM, SR designed and performed experiments, analysed data and wrote the manuscript; CP, AB, MMo, MD performed experiments; MMa, OR analysed data; all the authors reviewed the manuscript.

## Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

## Declaration of Competing Interest

The authors declare no competing interests.

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