Journal of Breath Research

OPEN ACCESS

RECEIVED
15 January 2015

REVISED
4 March 2015

ACCEPTED FOR PUBLICATION
12 March 2015

PUBLISHED
14 May 2015

Content from this work may be used under the terms of the Creative Commons Attribution 3.0 licence.

Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.

PAPER

Physiological variability in volatile organic compounds (VOCs) in exhaled breath and released from faeces due to nutrition and somatic growth in a standardized caprine animal model[4]

Sina Fischer[1], Phillip Trefz[2], Andreas Bergmann[2], Markus Steffens[2], Mario Ziller[3], Wolfram Miekisch[2], Jochen S Schubert[2], Heike Köhler[1] and Petra Reinhold[1]

[1] Institute of Molecular Pathogenesis at 'Friedrich-Loeffler-Institut' (Federal Research Institute for Animal Health), Naumburger Str. 96a, 07743 Jena, Germany

[2] Department of Anaesthesia and Intensive Care, University of Rostock, Schillingallee 35, 18057 Rostock, Germany

[3] Workgroup Biomathematics at 'Friedrich-Loeffler-Institut' (Federal Research Institute for Animal Health), Südufer 10, 17493 Greifswald–Insel Riems, Germany

E-mail: petra.reinhold@fli.bund.de

Keywords: animal model, exhaled breath, faeces, GC–MS, goat, volatile organic compound (VOC)

Abstract

Physiological effects may change volatile organic compound (VOC) concentrations and may therefore act as confounding factors in the definition of VOCs as disease biomarkers. To evaluate the extent of physiological background variability, this study assessed the effects of feed composition and somatic growth on VOC patterns in a standardized large animal model. Fifteen clinically healthy goats were followed during their first year of life. VOCs present in the headspace over faeces, exhaled breath and ambient air inside the stable were repeatedly assessed in parallel with the concentrations of glucose, protein, and albumin in venous blood. VOCs were collected and analysed using solid-phase or needle-trap microextraction and gas chromatograpy together with mass spectroscopy. The concentrations of VOCs in exhaled breath and above faeces varied significantly with increasing age of the animals. The largest variations in volatiles detected in the headspace over faeces occurred with the change from milk feeding to plant-based diet. VOCs above faeces and in exhaled breath correlated significantly with blood components. Among VOCs exhaled, the strongest correlations were found between exhaled nonanal concentrations and blood concentrations of glucose and albumin. Results stress the importance of a profound knowledge of the physiological backgrounds of VOC composition before defining reliable and accurate marker sets for diagnostic purposes.

S Online supplementary data available from stacks.iop.org/JBR/9/027108/mmedia

1. Introduction

A considerable number of volatile organic compounds (VOCs) are generated by cells or microorganisms throughout the body during various metabolic and biochemical processes (Schulz and Dickschat 2007, Thorn and Greenman 2012) and can be detected in different biological specimens such as exhaled breath or faeces (de Lacy Costello *et al* 2014). Therefore, the clinical application of VOC analysis may be a promising tool for the non-invasive diagnosis of metabolic, inflammatory, or infectious diseases in humans (Spanel and Smith 2011, Buszewski *et al* 2013, Wang and Wang 2013) and animals (Dobbelaar *et al* 1996, Knobloch *et al* 2010, Purkhart *et al* 2011, Peled *et al* 2012, Ellis *et al* 2014).

Controlled sampling, reliable analysis, and unequivocal identification of VOC biomarkers and potential confounding substances are mandatory for the application of VOC trace analysis (typical concentrations are in the nmol L^{-1}–pmol L^{-1} range). Adapted microextraction methods such as solid-phase microextraction (SPME) (Arthur and Pawliszyn 1990) and needle-trap microextraction (NTME) (Mieth *et al*

[4] Part of the data was presented at the 8th International Conference on Breath Research and Cancer Diagnosis (Breath Analysis 2014), Torun (Poland), 6–9th July 2014. The relating poster was honoured with the 1. Prize (best scientific poster presentation).

© 2015 IOP Publishing Ltd

2009, Lord *et al* 2010) enable controlled collection and pre-concentration of VOCs from the headspace (gas phase above liquids or solid materials) above faeces as well as from the exhaled breath and from room air samples (Miekisch *et al* 2014). Subsequent analysis by means of gas chromatography together with mass spectrometry (GC–MS) enables unequivocal identification and quantification of volatile compounds.

From a biological perspective, significant alterations of the VOC profile in humans (Filipiak *et al* 2012, Miekisch *et al* 2012, de Lacy Costello *et al* 2014) as well as in animals (Elliott-Martin *et al* 1997, Turner *et al* 2012) may occur due to environmental influences (nutritional effects, air contaminants, husbandry) or conditions in the host itself (age, sex, species). Additionally, volatile compounds originating from exogenous sources can be absorbed into the body and thus contribute to the body's pool of VOCs that have been endogenously generated (Miekisch *et al* 2004, Pleil *et al* 2013). Thus, clinical studies are often hampered by non-avoidable, host-related, confounding factors that could lead to erroneous conclusions when searching for biomarkers indicating diseases (Filipiak *et al* 2012).

This study takes both the methodological requirements of pre-concentration and the need for standardized biological conditions into account in order to assess the 'background' variability of VOCs in healthy conditions. Significant and well-defined changes occur with respect to digestion and metabolism in goats when they develop from milk-suckling kids to ruminating goats fed exclusively with plant products (Van Soest 1994). Thus, we considered ruminants as biologically relevant large-animal models for the assessment of the physiological effects of different nutrition regimes, and we hypothesized that changing diet, i.e. the replacement of the predominant milk intake (in goat kids) with an increasing percentage of plant products through to pure plant-based nutrition (in adults), would significantly influence the emitted VOCs.

To verify this hypothesis, this study evaluated the variability in the *in vivo* VOC composition due to growth and metabolic effects in a caprine model under defined conditions with respect to nutrition regime, husbandry, and environmental influences. VOC concentration patterns were examined in the headspace above faeces and in the exhaled breath of young, healthy goats during their first year of life. Environmental volatile compounds present in room air were taken into account. Results of this study stress the importance of a profound knowledge of the physiological effects and nutritional/environmental backgrounds of VOC composition before defining reliable and accurate marker sets for diagnostic purposes.

2. Animals, materials and methods

2.1. Legislation and ethical approval

This study was carried out in strict accordance with the German Animal Welfare Act, and animals were housed in conformity with the guidelines for animal welfare set forth by the European Community. The study protocol was approved by the Committee for the Ethics of Animal Experiments and the Protection of Animals of the State of Thuringia, Germany (Permit Number: 04-001/11). All experiments were conducted under supervision of the authorized institutional Agent for Animal Protection. Every effort was made to minimize discomfort and suffering throughout the duration of the study.

2.2. Animals, housing conditions, nutrition regime and health status

Fifteen goats ('Thüringer Wald Ziege', 14 male, 1 female) were involved in this longitudinal prospective study. The animals originated from one farm, were colostrum-fed after birth, and were conventionally raised for the first few days. Aged eight to 16 d and weighing 1.5 to 4.4 kg (3.0 $\pm$ 0.7; mean $\pm$ SD), they were brought to the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institut, Jena, Germany), where they were kept in one group under uniform conditions (room climate: 20.4 $\pm$ 2.7 °C, 63 $\pm$ 6% relative humidity; mean $\pm$ SD). Routine microbiological screening confirmed the absence of relevant infections (*Salmonella, Mycoplasma, Pasteurella, Mycobacteria)*. They were bedded on straw that was completely renewed every two weeks. The nutrition regime changed from milk replacer provided in the first weeks of life to a purely plant-based diet (concentrated feed and hay), as shown in figure 1. Confirmed by daily clinical examination (general behaviour, appetite, rectal temperature, etc), all animals were apparently clinically healthy throughout the entire study. To prevent the influence of sex hormones on the study, the male goats were castrated at the age of eight weeks according to good veterinary practice. The one female goat did not generate any outliers or extreme values.

2.3. Study design

The duration of the study was about one year: the goats were followed from the 3rd to the 57th week of life (wl). Breath gas and faecal samples for VOC analyses were collected in parallel at seven time points, i.e. 4, 8, 21, 32, 36, 44, and 51 wl as shown in figure 1. Room air was simultaneously collected from the animals' stable.

In addition to daily clinical examination, the respiratory rate was recorded at rest twice a week from the 7th wl onwards, and the individual body mass was assessed in 2-week intervals. The averages of the obtained parameters were calculated for 3- or 4-week intervals corresponding to the sample collections for the VOC analyses. Within each interval, jugular venous blood samples were obtained from each goat in order to assess glucose, total protein, and albumin in peripheral blood.

Because goats included here served as healthy controls for another study (Krüger *et al* 2015), necropsies of individual goats on a randomized basis were obligatory

Age (wl)	5	10	15	20	25	30	35	40	45	50	55	57
VOC analysis (wl)	4	8	21	32	36	44	51					
Clinical examination & Blood collection	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Nutrition	MR	MR + CF	CF									
Included animals (n)	n=15	n=13	n=10	n=7								
Necropsy (n)	n=2	n=3	n=3	n=7								

Figure 1. In the large-animal model, young, healthy goats were observed during the first year of their life. The animals were reared indoors under standardized conditions (housing and nutrition). VOC = volatile organic compound; wl = week of life; *n* = number of animals; MR = commercial milk replacer (3rd to 12th wl) (Denkamilk Capritop, Denkavit Futtermittel GmbH, Warendorf, Germany); CF = concentrated feed (6th to 57th wl) changed from a ration for lambs (Alleinfuttermittel für Ziegenmastlämmer, Landhandelsgesellschaft eG, Schmölln, Germany) to dairy concentrate (Milchleistungsfutter, Landhandelsgesellschaft eG, Schmölln, Germany) in the 15th wl; water and hay were offered *ad libitum.* For supplementation of minerals, lickstones were freely available in form of mineral blocks (Blattimin, Höveler Spezialfutterwerke GmbH and Co. KG, Dormagen, Germany).

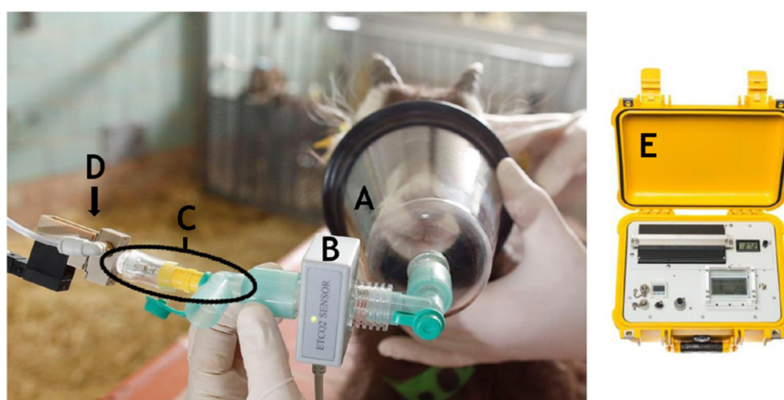

Figure 2. Automatic, CO_2-controlled sampling of exhaled breath in goats performed by means of needle-trap microextraction (NTME): A—face mask; B—CO_2 sensor of the capnometer; C—needle trap device (NTD), covered by a protective tube; D—CO_2-triggered flow valve; E—automatic alveolar sampling device connected to B and D.

and resulted in a decrease in the number of animals during the study (figure 1). Results of pathological examination confirmed the physical health of all 15 goats included.

2.4. Collection and processing of faecal samples

Faecal samples were collected on an individual basis directly in a clean sampling device before morning feeding; either through rectal manipulation (measuring rectal temperature) or during spontaneous defecation. Immediately after collection, about 3 g of fresh faeces per goat and time point were filled into a 20 mL headspace vial sealed with Teflon-coated rubber septa in combination with magnetic crimp caps (all: Gerstel GmbH and Co.KG, Muehlheim, Germany). The vials were stored at 4 °C and processed within 72 h after sampling.

For pre-concentration, gaseous samples were collected from the headspace above faeces using solid-phase microextraction (SPME). The sample vials were heated to 42 °C for 3 min, before the SPME fibre was pierced through the septum and exposed to the volatiles of the headspace for 7 min. The vial temperature was held at 42 °C for the duration of the exposure. SPME fibres (Supelco, Bellefonte, USA) consisting of PDMS Carboxen (75 μm) were used for all measurements. Before being used the first time, the SPME fibre was preconditioned in a GC injector at 300 °C for one hour and reconditioned at 290 °C in the GC injector for 30 min before each re-use. A blank run of the SPME fibre was performed every day before the measurements to ensure that the SPME coating was clean and that no uncontrolled bleeding took place. In addition, a gas standard of 2,3-dimethyl-1,3-butadiene in methanol was analysed at the beginning and end of every sampling queue to control performance of the extraction fibre.

2.5. Collection and processing of breath gas and room air samples

An automated sampling device (PAS Technology Deutschland GmbH, Magdala, Germany) combining mainstream capnometry and needle-trap microextraction (NTME) was used for breath gas sampling, as described previously (Trefz *et al* 2013b). In brief, this device enabled control of (i) sample volume and (ii) sampling conditions with respect to flow and pressure. Alveolar sampling was realized by connecting the sampling device to a mainstream capnometer (Capnoguard, Philips Respironics) and a

fast-responding, CO_2-triggered, flow valve (figure 2), and setting a threshold value for CO_2 of 25–30 mmHg (3.3–4.0 kPa). Above the threshold value the CO_2-triggered valve opened and predominantly alveolar gas was directed through the needle trap devices (NTDs) for collection of VOCs. As shown in figure 2, the system was adapted to spontaneously breathing conscious goats by a tightly fitting face mask (Henry Schein VET GmbH, Hamburg, Germany).

The flow rate during sampling was 20 mL min $^{-1}$ and total sample volume per goat per time point was set at 60 mL. The composition of the ambient air inside the animals' stable was observed by means of room air samples using NTME. At least two room air samples were collected per time point (one immediately before and one immediately after the breath gas measurements; 60 mL each) using the automated NTME sampling device without CO_2-controlled opening of the valve.

The single-bed, polymer-packed NTDs (Shinwa Ltd., Japan) were packed with 3 cm of a copolymer of methacrylic acid and ethylene glycol dimethacrylate. The NTDs were conditioned in a heating device (PAS Technology Deutschland GmbH, Magdala, Germany) at 195 °C for 20 h under permanent helium flow (1 bar) before first use, and re-conditioned at 195 °C for 30 min before they were applied for the pre-concentration of the samples. Each NTD was sealed by a Teflon cap (Shinwa LTD., Japan/PAS Technology Deutschland GmbH, Magdala, Germany) before and immediately after collecting a gaseous sample.

2.6. GC–MS analysis

VOC analyses were performed using GC–MS. An Agilent 7890 A gas chromatograph connected to an Agilent 5975 C inert XL mass selective detector (MSD) was used to separate and detect VOCs desorbed from SPME fibres, as described in Trefz *et al* (2013a). VOCs desorbed from the NTDs were separated by a gas chromatograph (Agilent 7890 A) and detected by a mass selective detector (Agilent 5975 C inert XL MSD), as previously described in Trefz *et al* (2013b).

2.7. Confirmation and quantification of potential marker substances with GC–MS

Substances were tentatively identified using a mass spectral library (NIST 2005 Gatesburg, PA, USA). In order to obtain unequivocal identification and quantification, the selected marker substances were verified by means of pure reference substances. For the calibration and determination of limit of detection (LOD, signal-to-noise ratio 3:1) and limit of quantification (LOQ, signal-to-noise ratio 10:1), different concentration levels of the reference substances were measured as previously described for SPME (Trefz *et al* 2013a) and NTME (Trefz *et al* 2012). Humidified standards were used for the quantification of NTME-GC–MS data. Substance identities as confirmed through retention time and mass spectra as well as quantitative parameters (LODs, LOQs) of reference substances are provided in supplementary table A1 (stacks.iop.org/JBR/9/027108/mmedia).

Acetone, 2-butanone, hexanal, nonanal, isoprene, and benzene were acquired from Ionimed Analytik GmbH (Innsbruck, Austria); butane, pentane, and hexane from Supelco (Bellefonte, USA); methyl isobutyl ketone, 1-propanol, styrene, 3-octanone, 2-heptanone, furan, heptane, methyl acetate, 2-methylfuran, 2-ethylfuran, 2-pentylfuran, 2-pentanone, 2-hexanone, 3-hexanone, 3-methyl-2-butanone, 3-methyl-2-pentanone, 2-propanethiol, benzaldehyde, 2,5-dimethylbenzaldehyde, dimethyltrisulfide and dimethylsulfide from Fluka/Sigma-Aldrich (Steinheim, Germany), 2-methylbutanal and dimethyldisulfide from Abbott GmbH and Co KG (Wiesbaden, Germany); ethylbenzene from Merck (Darmstadt, Germany); and 3-methylfuran from TCI Europe N.V. (Zwijndrecht, Belgium).

2.8. Assessment of blood parameters

The plasma concentration of glucose was analysed immediately after collecting heparinized blood (2.0 mL polypropylene syringes with lyophilized electrolyte-balanced heparin; PICO 50, Radiometer Medical ApS, Brønshøj, Denmark) using a combined blood-gas- and electrolyte analyser (ABL 725 Series, Radiometer Copenhagen, Copenhagen, Denmark) working with the manufacturer's standard electrodes.

The concentration of total protein in blood serum was measured spectrophotometrically (Cobas 6000, Roche/Hitachi, biuret method) and the concentration of serum albumin was derived by capillary electrophoresis (Capillarys 2, Sebia). Blood for serum production was collected with 9.0 mL syringes (S-Monovette, Sarstedt AG and Co. KG., Nuembrecht, Germany) and the serum harvested by centrifugation and stored at −20 °C until required for analysis.

2.9. Statistical analysis

PASW Statistics (Version 17.0, IBM Corporation, NY, USA), R (version 2.15.2; 2012-0-26, R Development Core Team) with package agricolae (version 1.1–3; 2012-12-21), Excel (Microsoft Excel, Excel 2010, Microsoft Corporation, USA), and Statgraphics Centurion XVI (Version 16.1.18, Statistical Graphics Corporation) were used for data analyses.

Normally distributed data is provided as mean ± standard deviation (SD) while data without normal or with unknown distribution is provided as median and range (difference between maximum and minimum). In box-and-whisker plots, outlier values (circles) were more than 1.5 times the length of a box away from the median. Extreme values (asterisks) are data beyond the three interquartile ranges.

The Mann–Whitney *U*-Test was used to identify significant differences between two groups of unpaired data. A multifactorial analysis of variance (ANOVA) was performed for the global test of whether any of

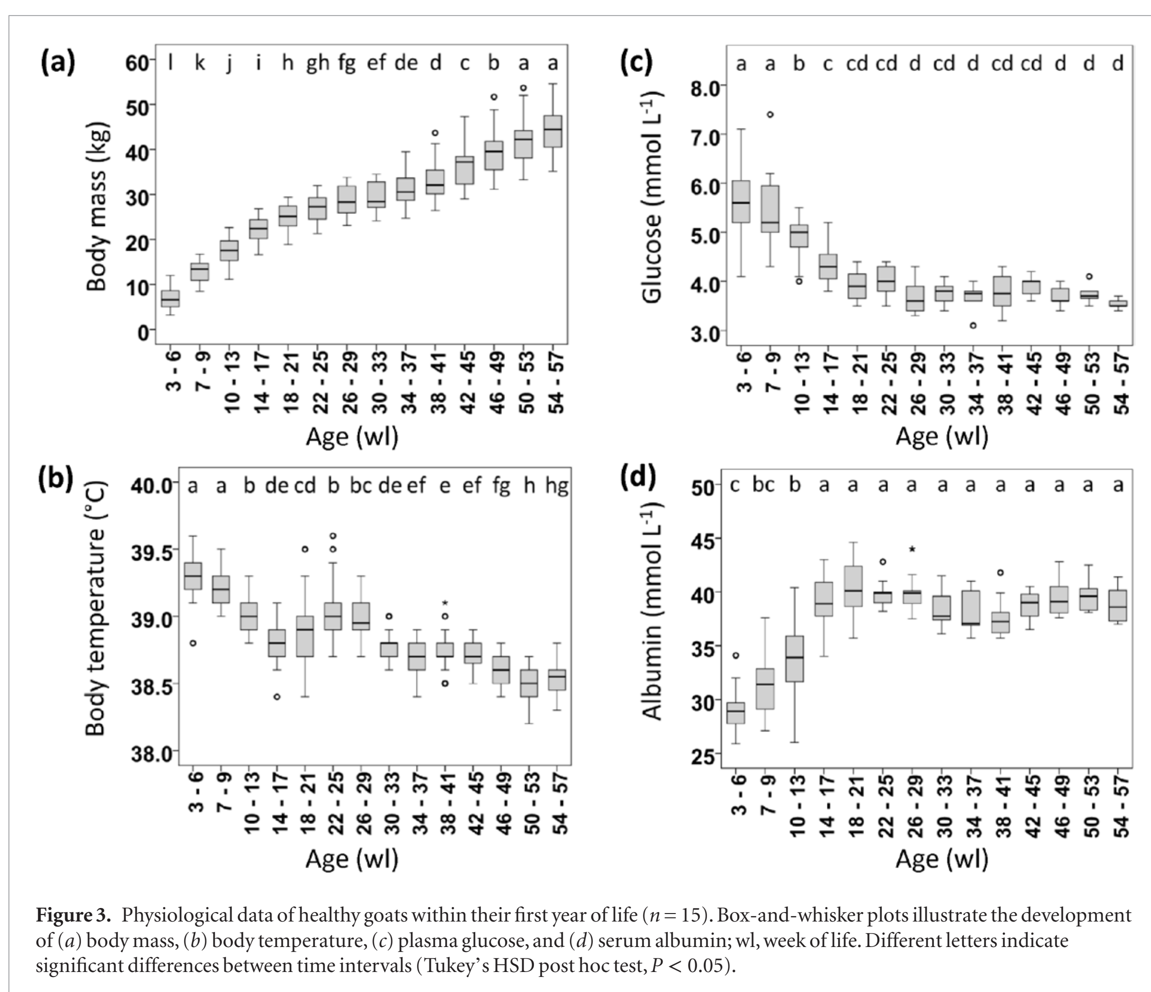

Figure 3. Physiological data of healthy goats within their first year of life ($n = 15$). Box-and-whisker plots illustrate the development of (*a*) body mass, (*b*) body temperature, (*c*) plasma glucose, and (*d*) serum albumin; wl, week of life. Different letters indicate significant differences between time intervals (Tukey's HSD post hoc test, $P < 0.05$).

the measured values differed in its expectations for some time intervals. An additional factor 'goat' was incorporated in the model to consider individual bindings of the data. The global significance level of 0.05 was adjusted according to the Bonferroni correction because of multiple testing of several features. For descriptive purposes, Tukey's HSD post hoc tests were applied for every feature to discern pairwise differences between specific time intervals. Significant differences are indicated using different letters starting with 'a' for the group with the highest responses. The strength of dependence between VOCs and blood components was assessed using regression analyses, and the best fitting model for each significant correlation was analysed. P-values < 0.05 were considered statistically significant for all statistical tests.

3. Results

3.1. Growth and development of the animals

In the developing healthy goats, body mass increased continuously with age (figure 3(*a*)), accompanied by a significant decrease in body temperature, although a slightly elevated body temperature was observed again during the summer months, corresponding to the 18th–29th wl (figure 3(*b*)). The respiratory rate at rest decreased significantly from 39.0 $\pm$ 10.6 breaths min^{-1} in goat kids aged 7–11 weeks to relatively constant values of 23.6 $\pm$ 2.0 breaths min^{-1} from the 14th wl onwards. The concentration of glucose in peripheral blood was highest in goat kids aged 3–6 weeks and decreased significantly until the 21st wl (figure 3(*c*)). Subsequently, constantly low concentrations of 3.8 $\pm$ 0.3 mmol L^{-1} were present until the end of the study. The concentration of total protein and albumin in peripheral blood increased significantly with increasing age until the 17th wl. While young goats aged 3–6 wl presented lowest concentrations of total protein (50.0 $\pm$ 5.6 g L^{-1}) and albumin (29.1 $\pm$ 2.2 g L^{-1}), higher and relatively constant concentrations of 67.1 $\pm$ 3.4 g L^{-1} (total protein) and 39.2 $\pm$ 2.0 g L^{-1} (albumin) were measured in venous blood of goats older than 18 weeks (all data: mean $\pm$ SD). Results for albumin are illustrated graphically in figure 3(*d*).

3.2. Volatile organic compounds in the headspace above faeces

Throughout the study period of one year, a marker set of 25 volatile substances was repeatedly assessed from 73 faecal samples by SPME-GC–MS measurements (table 1). The set included substances of the following chemical classes: ketones (10), aliphatic hydrocarbons (5), furans (5), sulfur-containing compounds (4), and esters (1). Figure 4(*a*) illustrates the courses of intensity for those 18 volatile substances presenting significant changes over time.

Table 1. Concentrations of VOCs assessed in the headspace above faeces.

VOCs		VOC concentrations (ppbV) Median (range)							ANOVA
		4 wl	8 wl	21 wl	32 wl	36 wl	44 wl	51 wl	*P*-value
		n = 10	*n* = 14	*n* = 15	*n* = 10	*n* = 10	*n* = 7	*n* = 7	
Ketones	Acetone	1386 (3659)^{a}	162 (569)^{b}	292 (278)^{b}	419 (271)^{b}	377 (308)^{b}	472 (505)^{b}	537 (377)^{b}	< 0.001
	2-Butanone	1553 (1719)^{a}	252 (814)^{b}	44 (94)^{b}	87 (106)^{b}	80 (113)^{b}	105 (128)^{b}	115 (98)^{b}	< 0.001
	2-Pentanone	6917 (16381)^{a}	724 (4701)^{b}	174 (186)^{b}	267 (112)^{b}	315 (166)^{b}	332 (375)^{b}	486 (268)^{b}	< 0.001
	2-Hexanone	2531 (13231)^{a}	276 (6462)^{b}	53 (228)^{b}	105 (64)^{b}	95 (103)^{b}	139 (173)^{b}	127 (107)^{b}	< 0.001
	3-Hexanone	263 (632)^{a}	238 (784)^{a}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	0 (79)^{b}	0 (0)^{b}	< 0.001
	2-Heptanone	136 (1081)	50 (585)	17 (12)	26 (14)	28 (21)	35 (39)	40 (51)	n.s.
	3-Octanone	66 (195)^{ab}	64 (287)^{ab}	0 (32)^{c}	83 (66)^{abc}	37 (26)^{bc}	79 (80)^{ab}	105 (85)^{a}	< 0.001
	Methyl isobutyl ketone	652 (881)^{a}	130 (225)^{b}	5 (8)^{b}	7 (4)^{b}	12 (8)^{b}	11 (11)^{b}	8 (5)^{b}	< 0.001
	3-Methyl-2-butanone	622 (1115)^{a}	136 (1389)^{b}	27 (163)^{b}	131 (189)^{b}	40 (48)^{b}	47 (135)^{b}	34 (80)^{b}	< 0.001
	3-Methyl-2-pentanone	3204 (33243)	1191 (33876)	11 (50)	16 (57)	27 (99)	25 (34)	18 (46)	n.s.
Aliphatic hydrocarbons	Butane	4 (12)^{a}	0 (0)^{b}	0 (0)^{b}	0 (4)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	< 0.001
	Pentane	50 (79)^{a}	19 (34)^{b}	12 (44)^{b}	15 (17)^{b}	19 (15)^{b}	12 (8)^{b}	28 (48)^{b}	< 0.001
	Hexane	27 (34)	13 (151)	0 (13)	9 (14)	0 (111)	8 (31)	9 (25)	n.s.
	Heptane	31 (54)^{a}	14 (24)^{b}	7 (7)^{b}	9 (8)^{b}	6 (4)^{b}	10 (4)^{b}	10 (7)^{b}	< 0.001
	Isoprene	5 (22)^{c}	6 (20)^{c}	8 (31)^{c}	4 (22)^{c}	14 (26)^{bc}	22 (44)^{a}	30 (30)^{ab}	< 0.001
Esters	Methyl acetate	2 (65)^{c}	6 (137)^{bc}	55 (69)^{ab}	54 (40)^{a}	52 (67)^{ab}	59 (64)^{a}	71 (65)^{a}	< 0.001
Sulfur containing compounds	2-Propanethiol	177 (1066)^{a}	7 (30)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	< 0.001
	Dimethylsulfide	2159 (27916)^{b}	11340 (47512)^{a}	76 (1969)^{b}	139 (449)^{b}	217 (329)^{b}	299 (934)^{b}	2502 (7424)^{b}	< 0.001
	Dimethyldisulfide	3217 (7084)	891 (48714)	0 (2)	1 (3)	0 (2)	0 (9)	4 (20)	n.s.
	Dimethyltrisulfide	50093 (124449)^{a}	7566 (86738)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	0 (82)^{b}	< 0.001
Furans	Furan	4 (9)^{a}	1 (2)^{b}	3 (9)^{ab}	2 (3)^{ab}	2 (2)^{b}	2 (2)^{b}	2 (1)^{b}	= 0.001
	2-Methylfuran	21 (77)	18 (34)	14 (25)	14 (18)	15 (11)	13 (6)	18 (9)	n.s.
	3-Methylfuran	148 (417)^{a}	106 (296)^{a}	17 (21)^{b}	26 (15)^{b}	21 (16)^{b}	20 (14)^{b}	25 (16)^{b}	< 0.001
	2-Ethylfuran	4 (11)	7 (11)	4 (6)	6 (5)	8 (6)	6 (3)	8 (5)	n.s.
	2-Pentylfuran	17 (2656)	23 (28)	17 (19)	27 (19)	29 (15)	24 (10)	38 (18)	n.s.

VOC, volatile organic compound; ppbV, parts per billion by volume; wl, week of life; n, number of animals; n.s., not significant.
For the ANOVA, the global significance level of 0.05 was adjusted according to Bonferroni. Different letters indicate significant differences between time intervals (Tukey's HSD post hoc test, $P < 0.05$. It was not possible to collect samples (faeces, exhaled breath) from each animal included in the study on all measurement campaigns for methodological reasons.

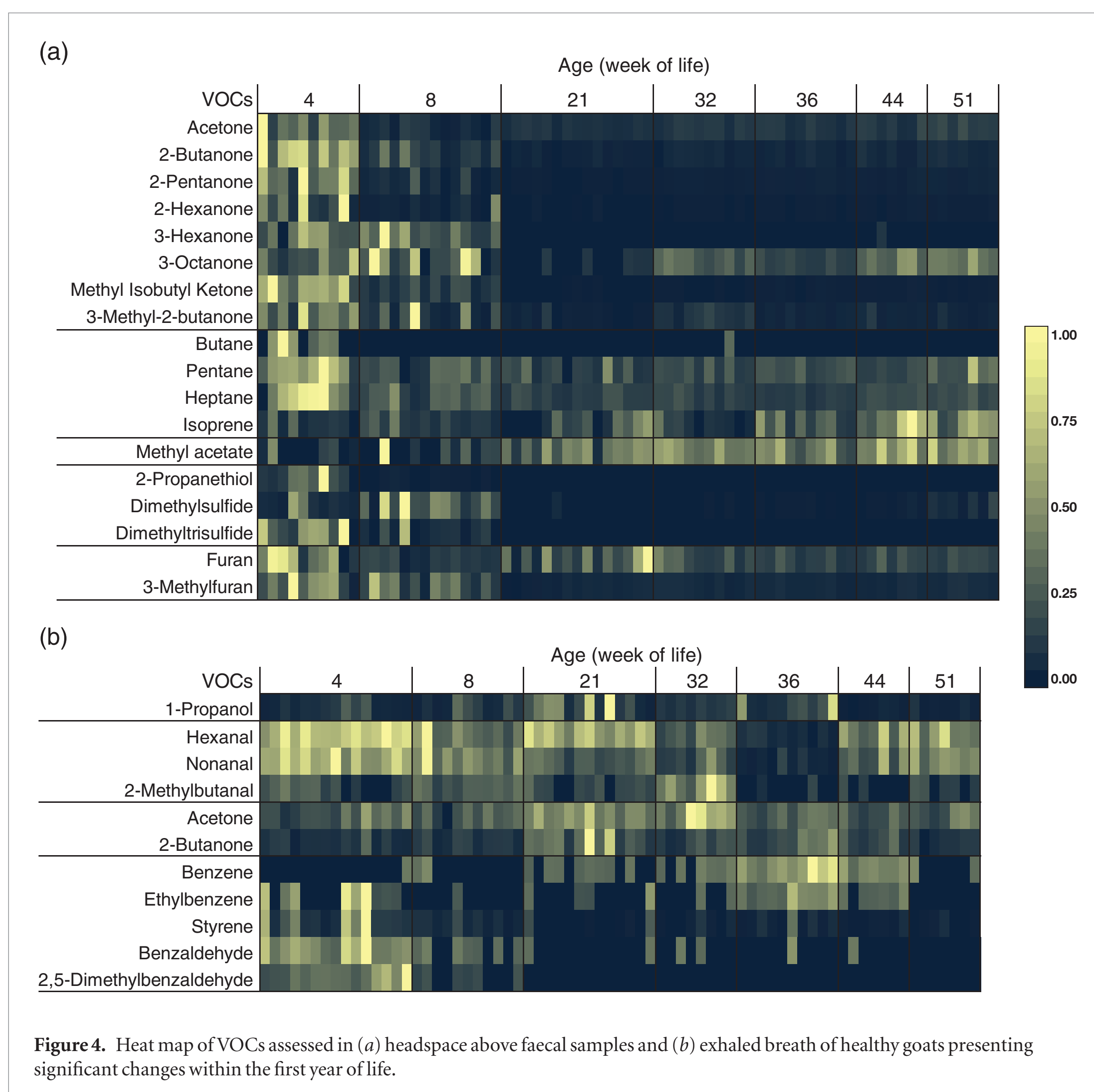

Figure 4. Heat map of VOCs assessed in (*a*) headspace above faecal samples and (*b*) exhaled breath of healthy goats presenting significant changes within the first year of life.

Detected concentrations of all observed ketones in the headspace above faecal samples decreased from the first (4th wl) to the third (21st wl) measurements. Concentrations of six (acetone, 2-butanone, 2-pentanone, 2-hexanone, methyl isobutyl ketone, and 3-methyl-2-butanone) out of 10 ketones were significantly higher in the first assessment when compared to subsequent measurements. In seven (acetone, 2-butanone, 2-pentanone, 2-hexanone, 3-hexanone, methyl isobutyl ketone, and 3-methyl-2-butanone) out of 10 ketones, there were no significant differences between the headspace concentrations from the 21st wl onwards. The headspace concentrations of the aliphatic hydrocarbons butane, pentane, and heptane were significantly higher at the first assessment when compared to subsequent measurements. In contrast, the detected concentrations of isoprene were significantly higher in the last two measurements (44th and 51st wl) when compared to the previous measurements. The headspace concentrations of methyl acetate increased sharply after the second measurements in the 8th wl. Headspace concentrations of furan and 3-methylfuran decreased significantly after the 4thwl and 8th wl respectively, while the other furans (2-methylfuran, 2-ethylfuran, and 2-pentylfuran) showed low variability between different measurements. The highest concentrations of all observed volatile sulfur compounds (VSCs) were detected in the 4th wl (2-propanethiol, dimethyldisulfide, and dimethyltrisulfide) and 8th wl (dimethylsulfide).

VOCs are plotted on the *y*-axis, while the different faecal headspace samples and breath samples, respectively, can be found on the *x*-axis. Measurements at different time points are separated by vertical lines. In order to emphasize the variation between the data and to show relative differences, data is normalized to the maximum concentrations determined in all samples.

3.3. Volatile organic compounds in the exhaled breath

Throughout the first year of the goats' lives, a set of eleven VOCs was repeatedly assessed in the 71 breath samples analysed (table 2). The substances belonged to the classes of aromatic hydrocarbons (5), aldehydes (3), ketones (2), and alcohols (1). The concentrations of all eleven volatile compounds in the exhaled breath changed significantly between the different time

Table 2. Concentrations of VOCs assessed in the exhaled breath.

VOCs		VOC concentrations (ppbV) Median (range)							ANOVA
		4 wl	8 wl	21 wl	32 wl	36 wl	44 wl	51 wl	
		$n = 15$	$n = 11$	$n = 13$	$n = 8$	$n = 10$	$n = 7$	$n = 7$	P-value
Alcohols	1-Propanol	186.78 (752.61)^{ab}	215.86 (909.85)^{ab}	634.15 (2857.24)^{a}	321.81 (216.10)^{ab}	466.45 (2331.00)^{ab}	52.35 (94.96)^{b}	68.92 (231.03)^{b}	< 0.001
Aldehydes	Hexanal	0.93 (0.43)^{a}	0.55 (0.69)^{bc}	0.81 (0.41)^{ab}	0.51 (0.28)^{cd}	0.32 (0.09)^{d}	0.61 (0.46)^{bc}	0.62 (0.48)^{bc}	< 0.001
	Nonanal	4.38 (3.44)^{a}	3.43 (4.40)^{ab}	2.36 (1.77)^{bc}	1.65 (2.67)^{cd}	0.94 (0.92)^{d}	2.24 (3.03)^{bc}	3.51 (2.10)^{ab}	< 0.001
	2-Methylbutanal	0.47 (0.93)^{bc}	0.66 (0.56)^{b}	0.38 (0.78)^{bc}	1.16 (1.79)^{a}	0 (0.38)^{c}	0 (0.48)^{c}	0.29 (0.58)^{bc}	< 0.001
Ketones	Acetone	24.25 (30.98)^{b}	26.37 (23.56)^{b}	40.24 (29.45)^{a}	46.93 (46.66)^{a}	29.54 (15.78)^{b}	23.34 (15.43)^{b}	26.73 (22.68)^{b}	< 0.001
	2-Butanone	0.41 (0.73)^{c}	0.5 (0.37)^{bc}	1 (2.17)^{a}	0.63 (0.52)^{bc}	0.76 (1.12)^{ab}	0.59 (0.59)^{bc}	0.44 (0.31)^{c}	< 0.001
Aromatic hydrocarbons	Benzene	0 (0.70)^{d}	0 (0.81)^{cd}	0.52 (0.67)^{cd}	0.59 (0.74)^{bc}	1 (1.15)^{a}	0.78 (0.47)^{ab}	0 (0.83)^{cd}	< 0.001
	Ethylbenzene	0.11 (0.55)^{ab}	0 (0.14)^{c}	0 (0.30)^{bc}	0 (0.19)^{bc}	0.22 (0.25)^{a}	0.15 (0.24)^{abc}	0 (0)^{c}	< 0.001
	Styrene	1.45 (11.95)^{a}	0.61 (2.24)^{b}	0.03 (3.12)^{b}	0.12 (1.09)^{b}	0.21 (4.32)^{b}	0.13 (0.65)^{b}	0.06 (0.70)^{b}	< 0.001
	Benzaldehyde	0.49 (0.69)^{a}	0.28 (0.40)^{b}	0 (0.36)^{b}	0 (0.38)^{b}	0 (0.50)^{b}	0 (0.37)^{b}	0 (0)^{b}	< 0.001
	2,5-Dimethylbenzal-dehyde	0.25 (0.59)^{a}	0.11 (0.16)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	0 (0)^{b}	0 (s0)^{b}	< 0.001

VOC, volatile organic compound; ppbV, parts per billion by volume; wl, week of life; n, number of animals.
For the ANOVA, the global significance level of 0.05 was adjusted according to Bonferroni. Different letters within a row indicate significant differences between time points. (Tukey's HSD post hoc test, $P < 0.05$). It was not possible to collect samples (faeces, exhaled breath) from each animal included in the study on all measurement campaigns for methodological reasons.

points. Intensities of these VOCs in exhaled breath are illustrated in a heat map (figure 4(*b*)).

Acetone and 2-butanone increased significantly after the 8th wl and returned to baseline values at the end of the study. Highest concentrations of the aldehydes hexanal and nonanal were detected in the assessments in the 4th wl, whereas 2-methylbutanal concentrations were highest in the 32nd wl.

3.4. Correlations between VOCs and blood components

The relationship of exhaled VOCs and VOCs above faeces to blood components was investigated by multiple regression analysis.

In the headspaces above faeces, concentrations of 3-hexanone, 3-methylfuran, methyl isobutyl ketone, 2-butanone, 2-pentanone, and dimethylsulfide were significantly correlated to blood concentrations of glucose, albumin, and total protein (table 3). In the case of blood proteins, correlations of the given VOC were higher to albumin than to total protein. Thus, figure 5 illustrates three typical examples of the multiple interactions between VOCs and the concentrations of glucose and albumin in blood.

Concentrations of 3-methyl-2-pentanone, 3-methyl-2-butanone, methyl acetate, and 2-heptanone were significantly correlated to blood concentrations of glucose only (table 4). In contrast, concentrations of dimethyltrisulfide, heptane, hexane, and pentane were significantly associated with concentrations of proteins (both total protein and albumin) in blood, but not to glucose (table 5).

In exhaled breath, only the concentration of nonanal was significantly correlated with blood concentrations of glucose, albumin, and total protein (table 3, figure 6). Concentrations of 2,5-dimethylbenzaldehyde and benzene in exhaled breath were significantly related to blood concentrations of glucose only (table 4), while concentrations of benzaldehyde and styrene were only correlated to concentrations of proteins (i.e. total protein and albumin) in blood (table 5).

3.5. Volatile organic compounds in room air

VOC concentrations assessed in room air samples are shown in comparison to concentrations in exhaled breath in table 6. Concentrations of styrene, 2,5-dimethylbenzaldehyde, and acetone in exhaled breath exceeded those in room air. Concentrations of benzene, ethylbenzene, benzaldehyde, hexanal, nonanal, 2-methylbutanal, 2-butanone, and 1-propanol present in the room air were in the same order of magnitude or higher when compared to concentrations detected in the exhaled breath.

4. Discussion

4.1. Validity and biological relevance of the model

Variances due to nutrition and metabolism in *in vivo* VOC profiles can impede the search for volatile biomarkers for diagnostic purposes but have hardly been addressed in literature before. Theoretically, nutrition may impact the excreted VOCs (i) as by- or end products of digestive processes of nutrients or food additives, or (ii) through diet-related influences of the gastrointestinal microbiota and subsequent changes in their metabolism. The large-animal model exploited for this study was considered the most useful to evaluate significant effects of different endogenous digestion profiles and associated changes in metabolism on volatile substances present in the metabolome.

The age dependence of physiological variables and metabolic development was assessed in growing goats developing from milk-suckling goat kids of about 6.6 kg to adults weighing about 45.5 kg and being fed by plant products only. Body mass, body temperature, and respiratory rate decreased physiologically with age, indicating that young goat kids have the highest metabolic rate. Despite still depending on milk, the pre-ruminant goats acquire the ability to gain nutrients from the fibrous material of plants due to microbial fermentation in the gastrointestinal tract. Becoming ruminants, cellulose and other complex carbohydrates are degraded by the gastrointestinal microbiota into volatile fatty acids (VFAs, e.g. acetate, propionate and butyrate) that replace glucose as the primary source of energy (Weimer 1992, Van Soest 1994). The decrease in plasma glucose in the first few weeks correlates with the increasing concentration of VFAs in blood (McCarthy and Kesler 1956). With maturation of the liver function, concentrations of both total protein and albumin in blood increase physiologically in goats during early life (Piccione *et al* 2011).

The strength of this study was that all involved subjects were kept under standardized conditions with respect to nutrition regime and ambient conditions during the entire time period of about one year. Thus, possible confounding factors present in clinical studies in humans (individual habits of diet, personal lifestyle, smoking behaviour, drugs, various environmental exposures, etc) were excluded. Due to this high level of standardization and a large homogeneity within the group of animals, we hypothesize that inter-individual variability observed in this study reflects the minimum of biological variability that has to be taken into account due to physiological inter-subject differences.

Influences of climate or season could not be completely avoided, as seen in the slightly higher body temperatures recorded during the summer period with extremely high ambient temperatures that could not be completely equilibrated by the air-conditioning system. Although this effect could have influenced the respiratory pattern (i.e. reduced tidal volume and increased respiratory rate), we have not considered it as a confounding factor because the CO_2-controlled sampling system enabled a standardized collection of 60 mL exhaled breath (per goat per time point), derived mainly from the alveolar region.

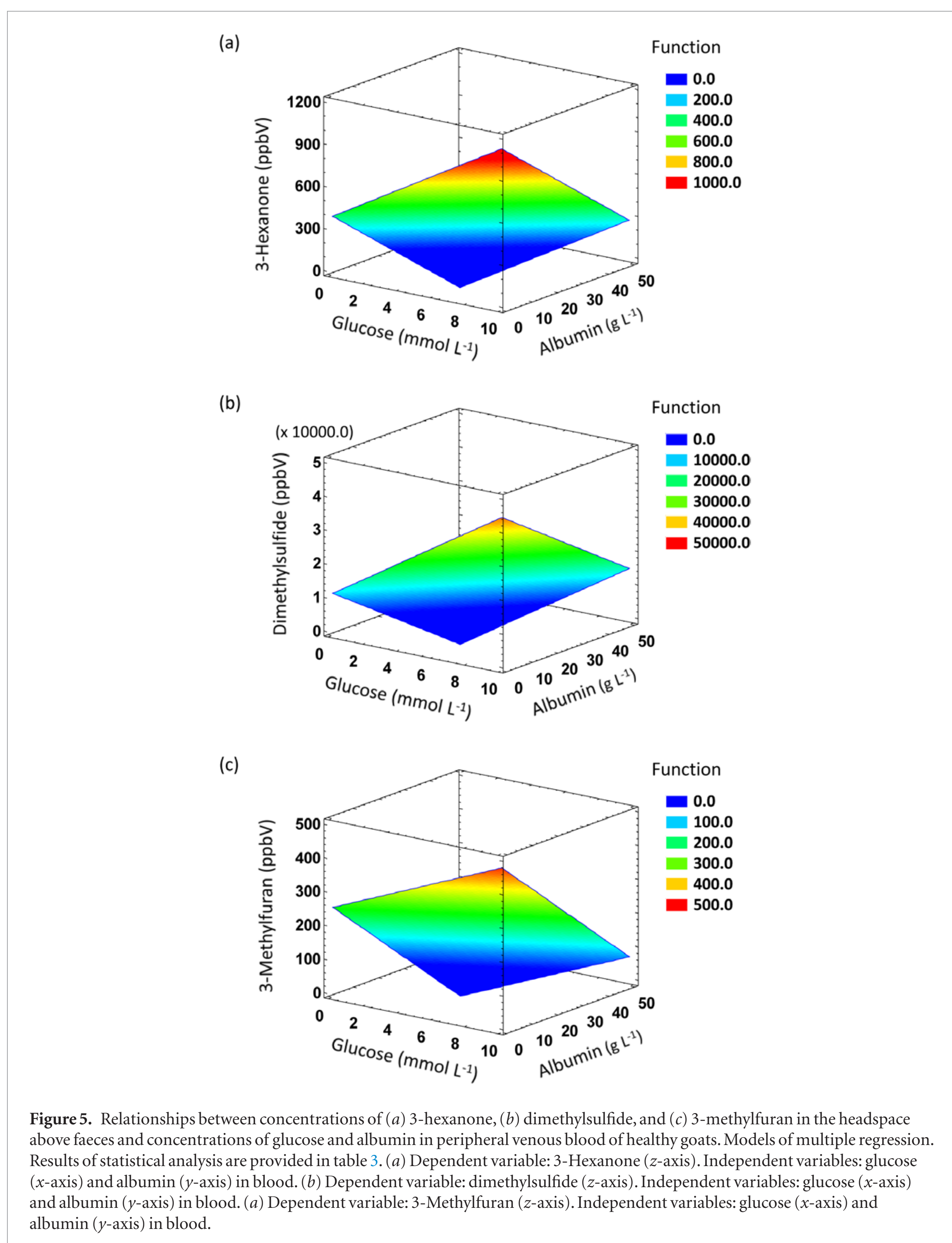

Figure 5. Relationships between concentrations of (*a*) 3-hexanone, (*b*) dimethylsulfide, and (*c*) 3-methylfuran in the headspace above faeces and concentrations of glucose and albumin in peripheral venous blood of healthy goats. Models of multiple regression. Results of statistical analysis are provided in table 3. (*a*) Dependent variable: 3-Hexanone (*z*-axis). Independent variables: glucose (*x*-axis) and albumin (*y*-axis) in blood. (*b*) Dependent variable: dimethylsulfide (*z*-axis). Independent variables: glucose (*x*-axis) and albumin (*y*-axis) in blood. (*a*) Dependent variable: 3-Methylfuran (*z*-axis). Independent variables: glucose (*x*-axis) and albumin (*y*-axis) in blood.

4.2. VOCs assessed in the headspace over faeces

Faeces are a dynamic mixture of residues of food intake, digestive and excretory processes, and epithelial cells as well as fragments and metabolites of the gastrointestinal microbiota (Piatkowski *et al* 1990). Thus, several volatile substances emitted from faeces have to be regarded as end or by-products of the host's metabolism or bacteria present in the gastrointestinal tract. Slight changes in one of these components, i.e. host, diet, or gastrointestinal microbiota, might be reflected by changes in the VOC profile.

In our study, concentrations of 18 out of 25 selected VOCs present in the headspace above faeces varied at different time points throughout the study. During the milk-suckling phase, highest concentrations of most identified ketones, aliphatic hydrocarbons, furans, and sulfur-containing compounds were emitted and decreased to lower concentrations in the advanced ruminant, whereas emission of other volatile compounds such as isoprene and methyl acetate increased with age.

The diversity and metabolic type of species constituting the gastrointestinal microbiota of ruminants change with increasing age (Draksler *et al* 2002) and adapt dynamically to environmental conditions, e.g. change of nutrition (Callaway *et al* 2010, Metzler-Zebeli

Table 3. Multiple interactions between VOCs assessed in headspaces above faeces or exhaled breath, respectively, and blood components in healthy goats.

Equations of multiple regression analysis	Model *P*-value (ANOVA)	Gluc. *P*-value	Alb. *P*-value	Prot. *P*-value	R^2 (%)	DW *P*-value
VOCs assessed in headspace above faeces (*n* = 71)						
3-Hexanone [ppbV] = 383.71 + 72.93 × Glucose [mmol L^{-1}] − 16.75 × Albumin [g L^{-1}]	0.000	0.001	0.000		59.89	0.628
3-Hexanone [ppbV] = 236.68 + 79.37 × Glucose [mmol L^{-1}] − 7.89 × Protein total [g L^{-1}]	0.000	0.001		0.002	56.77	0.684
3-Methylfuran [ppbV] = 252.27 + 22.05 × Glucose [mmol L^{-1}] − 8.03 × Albumin [g L^{-1}]	0.000	0.033	0.000		52.88	0.666
3-Methylfuran [ppbV] = 234.09 + 21.01 × Glucose [mmol L^{-1}] − 4.33 × Protein total [g L^{-1}]	0.000	0.055		0.000	51.65	0.714
Methyl isobutyl ketone [ppbV] = 684.61 + 66.06 × Glucose [mmol L^{-1}] − 23.62 × Albumin [g L^{-1}]	0.000	0.036	0.000		51.45	0.000
Methyl Isobutyl Ketone [ppbV] = 442.56 + 77.88 × Glucose [mmol L^{-1}] − 10.75 × Protein total [g L^{-1}]	0.000	0.024		0.003	47.17	0.000
2-Butanone [ppbV] = 779.55 + 211.21 × Glucose [mmol L^{-1}] − 38.20 × Albumin [g L^{-1}]	0.000	0.005	0.006		47.31	0.000
2-Butanone [ppbV] = 259.96 + 240.42 × Glucose [mmol L^{-1}] − 16.04 × Protein total [g L^{-1}]	0.000	0.003		0.045	44.46	0.000
2-Pentanone [ppbV] = 3688.44 + 1272.2 × Glucose [mmol L^{-1}] − 209.86 × Albumin [g L^{-1}]	0.000	0.020	0.039		35.26	0.013
2-Pentanone [ppbV] = 378.69 + 1468.57 × Glucose [mmol L^{-1}] − 83.27 × Protein total [g L^{-1}]	0.000	0.012		0.153	33.09	0.007
Dimethylsulfide [ppbV] = 11062.1 + 3286.0 × Glucose [mmol L^{-1}] − 577.91 × Albumin [g L^{-1}]	0.000	0.028	0.038		33.85	0.462
Dimethylsulfide [ppbV] = 9731.78 + 3213.28 × Glucose [mmol L^{-1}] − 311.65 × Protein total [g L^{-1}]	0.000	0.041		0.050	33.42	0.525
VOC assessed in exhaled breath (*n* = 69)						
Nonanal [ppbV] = 5.06 + 0.44 × Glucose [mmol L^{-1}] − 0.11 × Albumin [g L^{-1}]	0.000	0.030	0.005		40.93	0.004
Nonanal [ppbV] = 4.15 + 0.49 × Glucose − 0.06 × Protein total [g L^{-1}]	0.000	0.022		0.020	38.75	0.003

Dependent variable: VOC. Independent variables: blood concentrations of glucose (Gluc.) and albumin (Alb.) or total protein (Prot.), respectively. Since the *P*-value in the ANOVA table is less than 0.05, there is a statistically significant relationship between the three variables per equation at the 95.0% confidence level. *P*-values less than 0.05 for each independent variable indicate that the given variable contributes statistically significant to the regression model at the 95.0% confidence level. The R^2-statistic indicates how strong the model as fitted explains the variability in the given VOC. The Durbin–Watson (DW) statistic tests the residuals for any significant correlation. With a *P*-value greater than 0.05, there is no indication of serial autocorrelation in the residuals at the 95.0% confidence level. With a *P*-value less than 0.05, there is an indication of possible serial correlation at the 95.0% confidence level.

Table 4. Regressions between VOCs assessed in headspaces above faeces or exhaled breath, respectively, and blood concentrations of glucose in healthy goats.

Equations of simple regression analysis	Best fitting model	Model *P*-value (ANOVA)	*r*	R^2 (%)	Durbin–Watson *P*-value
VOCs assessed in headspace above faeces ($n = 71$)					
3-Methyl-2-pentanone [ppbV] = exp(−5.81 + 2.38 × Glucose [mmol L⁻¹])	$Y = \exp(a + b \times X)$ (exponential)	0.000	0.861	74.074	0.004
3-Methyl-2-butanone [ppbV] = −302.99 + 24.24 × Glucose [mmol L⁻¹]^2	$Y = a + b \times X$^2 (squared-*X*)	0.000	0.784	61.513	0.457
Methyl acetate [ppbV] = (−4.8 + 45.66/ Glucose [mmol L⁻¹])^2	$Y = (a + b/X)$^2 (square root-*Y* reciprocal-*X*)	0.000	0.614	37.641	0.722
2-Heptanone [ppbV] = (0.12 + 0.36 × Glucose [mmol L⁻¹]^2)^2	$Y = (a + b \times X$^2)^2 (square root-*Y* squared-*X*)	0.000	0.593	35.208	0.218
VOCs assessed in exhaled breath ($n = 69$)					
2.5-Dimethylbenzaldehyde [ppbV] = (−1.07 + 0.83 × ln(Glucose [mmol L⁻¹]))^2	$Y = (a + b \times \ln(X))$^2 (square root-*Y* logarithmic-*X*)	0.000	0.724	52.380	0.000
Benzene [ppbV] = (−0.85 + 5.56/ Glucose [mmol L⁻¹])^2	$Y = (a + b/X)$^2 (square root-*Y* reciprocal-*X*)	0.000	0.538	28.961	0.004

Dependent variable: VOC. Independent variable: blood concentrations of glucose. Since the *P*-value in the ANOVA table is less than 0.05, there is a statistically significant relationship between the two variables at the 95.0% confidence level. The correlation coefficient (*r*) indicates the strength of relationship between the variables. The R^2-statistic indicates how strong the model explains the variability in the given VOC. The Durbin–Watson (DW) statistic tests the residuals for any significant correlation. With a *P*-value greater than 0.05, there is no indication of serial autocorrelation in the residuals at the 95.0% confidence level. With a *P*-value less than 0.05, there is an indication of possible serial correlation at the 95.0% confidence level.

et al 2013, Kim *et al* 2014). Ketones and aliphatic hydrocarbons can derive from fatty acids due to bacterial conversion (Miekisch *et al* 2004, Schulz and Dickschat 2007, Thorn and Greenman 2012, de Lacy Costello *et al* 2014) but are also known to be generated endogenously in the host during fat and carbohydrate metabolism (Garner *et al* 2007, Schubert *et al* 2012) or peroxidation of fatty acids (Miekisch *et al* 2004). The appearance of isoprene in faeces is likely due to bacterial generation (Kuzma *et al* 1995) but it is also produced during the endogenous cholesterol biosynthesis (Deneris *et al* 1985), mainly formed in the intestinal mucosa. Endogenous production of esters such as methyl acetate from VFAs is assumed and might reflect gut wall esterase activity as well as bacterial esterase activity (Garner *et al* 2007). Changes in the composition of VSCs in the headspace over faeces can be directly related to the intestinal flora and may rather be associated with changes in the overall number than with the balance of the gastrointestinal microbiota (Garner *et al* 2007, Hales *et al* 2012, Walton *et al* 2013).

We also identified quantitative relationships between VOCs in the headspace above faeces and blood components. Although determination of the biochemical origin of VOCs was not possible in this study, results indicate a potential endogenous contribution to the VOC profile emitted by faeces which remains to be elucidated in future studies.

In our study the major changes in the headspace VOC composition occurred during the first three measurements, with the change of nutrition. Therefore, the physical development of the host, including the digestive tract, ingredients of diet as well as composition of the gastrointestinal microbiota, are most likely major factors influencing the VOC concentration pattern emitted by faeces. Composition of the gastrointestinal microbiota varies between species due to different physiology of digestion (ruminant versus monogastric animals). Although the stomach of humans as monogastric organisms contains only very few bacteria (Guarner and Malagelada 2003), complex and dynamic microbiota inhabit the large intestine in high densities (Simon and Gorbach 1984). Thus, the composition of both nutrition and intestinal flora might significantly influence VOCs released from stool in humans, too.

4.3. VOCs assessed in exhaled breath

CO_2-controlled breath sampling (Trefz *et al* 2013b) enabled collection of VOCs present in alveolar gas. It is generally accepted that this portion of exhaled breath reflects most closely the composition of VOCs originating from blood (Miekisch *et al* 2004, Beauchamp 2011). Similar to VOCs emitted by faeces, variances in the composition of exhaled VOCs can result from physiological factors such as growth effects and metabolic development as well as from changing

Table 5. Regressions between VOCs assessed in headspaces above faeces or exhaled breath, respectively, and blood concentrations of proteins in healthy goats.

Equations of regression	Best fitting model	Model P-value (ANOVA)	r	R^2 (%)	Durbin–Watson P-value
VOCs assessed in headspace above faeces (n = 71)					
Dimethyltrisulfide [ppbV] = (−408.88 + 16188/Albumin [g L^{-1}])^2	$Y = (a + b/X)$^2(square root-Y reciprocal-X)	0.000	0.752	56.58	0.719
Dimethyltrisulfide [ppbV] = (−345.8 + 23740.6/Protein total[g L^{-1}])^2		0.000	0.705	49.64	0.475
Heptane [ppbV] = −33.22 + 1614.57/Albumin [g L^{-1}]	$Y = a + b/X$(reciprocal-X)	0.000	0.607	36.80	0.000
Heptane [ppbV] = −31.67 + 2656.15/Protein total [g L^{-1}]		0.000	0.637	40.63	0.000
Hexane [ppbV] = (−5.84 + 291.5/Albumin [g L^{-1}])^2	$Y = (a + b/X)$^2 (square root-Y reciprocal-X)	0.000	0.513	26.27	0.373
Hexane [ppbV] = (−6.05 + 509.04/Protein total [g L^{-1}])^2		0.000	0.572	32.68	0.541
Pentane [ppbV] = −29.09 + 1838.72/Albumin [g L^{-1}]	$Y = a + b/X$ (reciprocal-X)	0.000	0.467	21.79	0.001
Pentane [ppbV] = −29.13 + 3134.06/Protein total [g L^{-1}]		0.000	0.508	25.82	0.001
VOCs assessed in exhaled breath (n = 69)					
Benzaldehyde [ppbV] = −1.03 + 42.30/Albumin [g L^{-1}]	$Y = a + b/X$ (reciprocal-X)	0.000	0.761	57.88	0.320
Benzaldehyde [ppbV] = −0.85 + 61.4/Protein total [g L^{-1}]		0.000	0.684	46.80	0.106
Styrene [ppbV] = (−2.36 + 109.56/Albumin [g L^{-1}])^2	$Y = (a + b/X)$^2 (square root-Y reciprocal-X)	0.000	0.644	41.52	0.556
Styrene [ppbV] = (−1.90 + 159.32/Protein total [g L^{-1}])^2		0.000	0.581	33.70	0.342

Dependent variable: VOC. Independent variable: blood concentrations of total protein or albumin, respectively. Since the P-value in the ANOVA table is less than 0.05, there is a statistically significant relationship between the two variables at the 95.0% confidence level. The correlation coefficient (r) indicates the strength of relationship between the variables. The R^2-statistic indicates how strong the model explains the variability in the given VOC. The Durbin–Watson (DW) statistic tests the residuals for any significant correlation. With a P-value greater than 0.05, there is no indication of serial autocorrelation in the residuals at the 95.0% confidence level. With a P-value less than 0.05, there is an indication of possible serial correlation at the 95.0% confidence level.

nutrition. Substances generated in the alimentary tract can be absorbed through the intestinal mucosa, distributed via the bloodstream, and exhaled through the lungs (Baranska *et al* 2013).

A significant increase in acetone and 2-butanone occurred after weaning from milk replacer in the 12th wl. Both substances were detected in the breath of cattle before this and were attributed to fatty-acid degradation during digestion (Elliott-Martin *et al* 1997). Fatty acids are products of the increasing bacterial fermentation by the gastrointestinal microbiota. Weaning of goats was followed by a significant increase in acetone in the exhaled breath in the 21st and 32nd wl. Weaning can result in a transient period with lack of energy and can therefore cause an increase in lipolysis and subsequently ketogenesis with elevated levels of ketone bodies in blood such as acetoacetate, ß-hydroxybutyrate, and acetone (Schultz 1968). Elevated levels of aldehydes such as hexanal, nonanal, and 2-methylbutanal in exhaled breath were associated with endogenous generation by protein oxidation or lipid peroxidation of unsaturated fatty acids due to oxidative stress (Miekisch *et al* 2004). Generation by bacteria of the gastrointestinal microbiota is a potential source of aldehydes, as emission is reported for *Proteus* (Aarthi *et al* 2014) and *Staphylococcus* (Beck *et al* 2002).

Concentrations of nonanal assessed in exhaled breath were associated with higher concentrations of glucose and lower concentrations of proteins in blood. Additionally, we found a quantitative relationship between concentrations of both 2,5-dimethylbenzaldehyde and benzene and glucose, and, in contrast, between concentrations of both benzaldehyde and styrene and proteins (albumin, total protein). Serum albumin enhances the aqueous solution of numerous substrates through non-specific binding and acts as the major transport protein in plasma for multiple molecules (Varshney *et al* 2010). Fatty acids can increase their concentrations in plasma by binding to albumin (van der Vusse 2009). Changes in the serum albumin concentration may alter the binding capacity of molecules, depending on their chemical properties, and therefore potentially have an impact on the composition and concentration of blood-borne VOCs in exhaled breath.

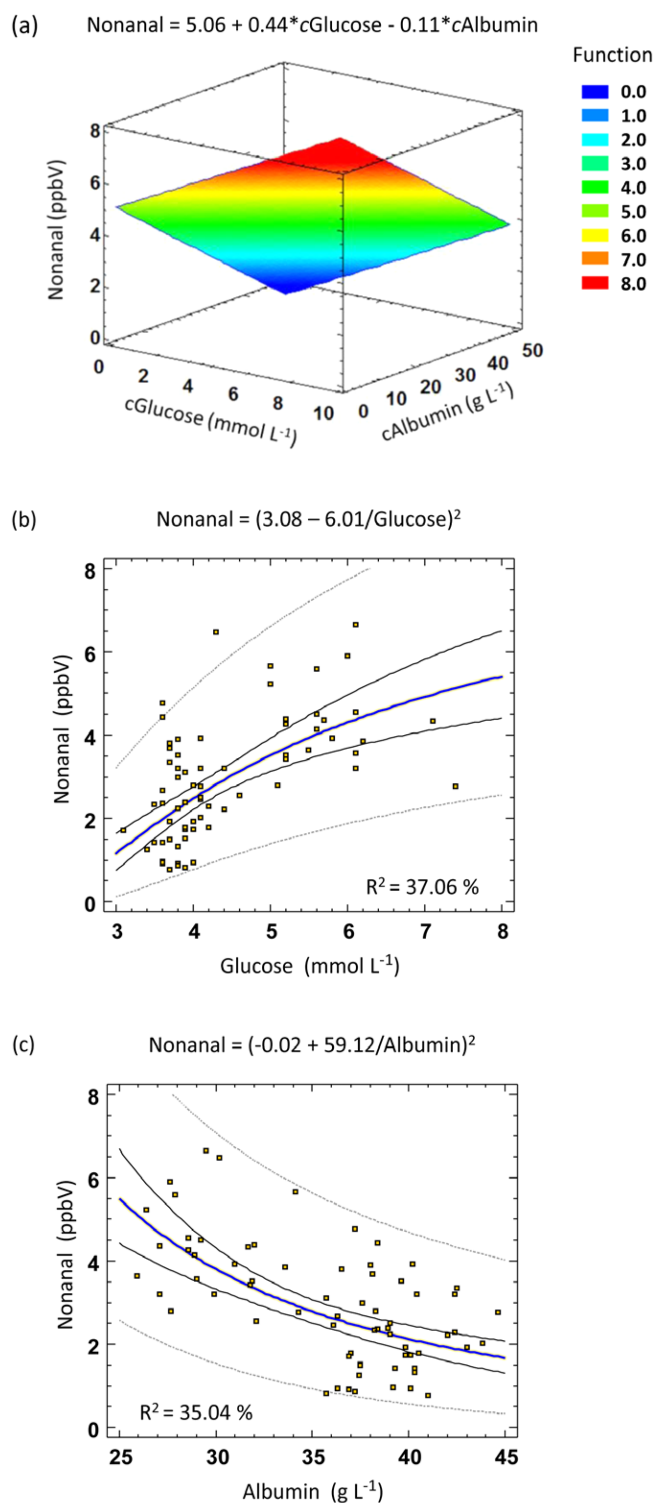

Figure 6. Relationships between concentrations of nonanal in exhaled breath and concentrations of glucose and/or albumin, respectively, in peripheral venous blood of healthy goats. (*a*) Model of multiple regression. Dependent variable: nonanal (*z*-axis). Independent variables: glucose (*x*-axis) and albumin (*y*-axis) in blood. Results of statistical analysis are provided in table 3. (*b*) Model of simple regression between nonanal (dependent variable; *Y*) and glucose (independent variable; *X*). Best fitting model: $Y = (a + b / X)$ ^2 ($R^2 = 37.06\%$, $P < 0.000$). Symbols represent single measurements ($n = 69$). The plot shows the regression line (blue, solid) and two sets of limits. The inner limits (black, solid) provide 95% confidence intervals for the mean value of *Y* at any selected *X*. The outer lines (grey, broken) are 95% prediction limits for new observations. (*c*) Model of simple regression between nonanal (dependent variable; *Y*) and Albumin (independent variable; *X*). Best fitting model: $Y = (a + b / X)$ ^2 ($R^2 = 35.04\%$, $P < 0.000$). Symbols represent single measurements ($n = 69$). The plot shows the regression line (blue, solid) and two sets of limits. The inner limits (black, solid) provide 95% confidence intervals for the mean value of *Y* at any selected *X*. The outer lines (grey, broken) are 95% prediction limits for new observations.

In general, the strength of correlations (R^2) between blood components and VOCs assessed in the alveolar phase of exhaled breath or VOCs in the headspace over faeces, respectively, was comparable or slightly lower for exhaled VOCs. Consequently, this study provides strong evidence that the metabolic status of the

Table 6. Comparison of VOCs in exhaled breath and in room air.

VOCs		VOC concentrations (ppbV) Median (range) Exhaled Breath $n = 71$	Room air $n = 20$	Mann-Whitney U-Test *P*-value
Aromatic hydrocarbons	Benzene	0.51 (1.76)	0.87 (5.33)	< 0.001
	Ethylbenzene	0 (0.55)	0 (0.55)	n.s.
	Styrene	0.26 (12.21)	0.05 (1.16)	< 0.001
	Benzaldehyde	0 (0.96)	0 (0.39)	n.s.
	2,5-Dimethyl-benzaldehyde	0 (0.73)	0 (0)	< 0.01
Aldehydes	Hexanal	0.68 (0.85)	1.28 (1.73)	< 0.001
	Nonanal	2.79 (5.85)	4.87 (13.2)	< 0.001
	2-Methylbutanal	0.38 (2.36)	0.61 (3.01)	< 0.05
Ketones	Acetone	31.96 (61.21)	19.01 (19.31)	< 0.001
	2-Butanone	0.54 (2.39)	0.9 (1.67)	=0.001
Alcohols	1-Propanol	218.6 (2903.88)	390.38 (3369.97)	=0.01

VOC, volatile organic compound; n, number of measurements; ppbV, parts per billion by volume; n.s., not significant.

organism contributes significantly to all VOC profiles emitted at different sites of the body.

4.4. Room air

Environmental volatile compounds can significantly contribute to the body's pool of VOCs through inhalation, ingestion, and percutaneous or mucosal absorption (Miekisch *et al* 2004, Pleil *et al* 2013). In agreement with our study, Mochalski *et al* (2013) found higher concentrations of 2-butanone in room air when compared with exhaled breath, and suggested a contribution of inhaled exogenous 2-butanone to blood levels. Aromatic hydrocarbons are common environmental contaminants (Filipiak *et al* 2012) and exhalation was reported in several prior studies (Phillips *et al* 1999, Pleil 2009). In our study, concentrations of styrene in exhaled breath were significantly higher compared to concentrations measured in room air. Styrene was previously detected in the breath of healthy human volunteers (Phillips *et al* 1999) and healthy cattle (Spinhirne *et al* 2004). Possible sources of exhaled styrene are intake via contaminated food (e.g. milk replacer) or absorption from plastic materials (Varner and Breder 1981), e.g. via the teat used for suckling milk replacer since endogenous generation of styrene is so far unknown. Due to its high lipophilicity, styrene can accumulate in fat tissue and subsequently be released into breath (Withey 1978).

In our study, the concentrations of 1-propanol in room air were mostly higher than those measured in the exhaled breath. Since 1-propanol is a multipurpose solvent used in cleaner or disinfectants and use of disinfectants could not be completely avoided, intake through inhalation is possible and detection of 1-propanol in exhaled breath most likely represents exogenous contamination.

When sampling exhaled breath, the concurrent determination of the current composition of room air should be mandatory in order to gain knowledge about inhaled compounds (Miekisch *et al* 2012). Nevertheless, information about the environmental constituents is just a snapshot and the previous exposure history of contaminants remains undetermined.

As room air is a mixture of ambient air with emissions originating from the animal itself (breath and sweat) and its excretions (faeces and urine), potential endogenous volatile biomarkers can accumulate in room air. Room air samples could thus potentially be used for diagnostic purposes in the future.

5. Conclusions

This study revealed significant alterations in *in vivo* VOC profiles in both breath gas and faeces, most likely induced by metabolic effects in growing subjects. Results of a standardized large-animal model proved the impact of physiological variability on released VOCs at different sites of the body. Characterization of environmental compounds should be mandatory for validation of all studies that deal with breath gas analysis. Results emphasize the importance of a profound knowledge of physiological and exogenous influences on VOC composition before defining reliable and accurate marker sets for diagnostic purposes.

Acknowledgments

The authors are very grateful for the excellent assistance of Annelie Langenberg, Sylke Stahlberg, Ines Lemser, and all colleagues of the technical staff of the animal house at the Friedrich-Loeffler-Institut. We thank Lisa Rösner (University of Applied Sciences, Jena) and Klaus Klepik (University of Rostock) for technical assistance in conducting the experiment. We extend our thanks to Maria-Christina Haase for her help in providing literature and Wolfram Maginot for his

excellent photographic support (both FLI, Jena). We are also very thankful to Dietmar Hein from PAS Technology (Magdala, Germany) for his continuous technical support and to Eva Radtke and Antje Willing (Institut für Veterinärmedizinische Diagnostik GmbH, Berlin) for analysing the concentrations of total protein and albumin in blood. Finally, we wish to extend our gratitude to the anonymous referees for the careful reading of the manuscript.

This study was financially supported by Deutsche Forschungsgemeinschaft (DFG) under Grant no. RE 1098/4-1. The study was partially funded by the Animal Health Funds of Thuringia, Hesse, Mecklenburg-Western Pomerania, Lower Saxony, Rhineland-Palatinate, and Baden-Wuerttemberg. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

References

Aarthi R, Saranya R and Sankaran K 2014 2-methylbutanal, a volatile biomarker, for non-invasive surveillance of proteus *Appl. Microbiol. Biotechnol.* **98** 445–54

Arthur C L and Pawliszyn J 1990 Solid-phase microextraction with thermal-desorption using fused-silica optical fibers *Anal. Chem.* **62** 2145–8

Baranska A, Tigchelaar E, Smolinska A, Dallinga J W, Moonen E J, Dekens J A, Wijmenga C, Zhernakova A and Van Schooten F J 2013 Profile of volatile organic compounds in exhaled breath changes as a result of gluten-free diet *J. Breath Res.* **7** 037104

Beauchamp J 2011 Inhaled today, not gone tomorrow: pharmacokinetics and environmental exposure of volatiles in exhaled breath *J. Breath Res.* **5** 037103

Beck H C, Hansen A M and Lauritsen F R 2002 Metabolite production and kinetics of branched-chain aldehyde oxidation in staphylococcus xylosus *Enzyme Microbial Technol.* **31** 94–101

Buszewski B, Grzywinski D, Ligor T, Stacewicz T, Bielecki Z and Wojtas J 2013 Detection of volatile organic compounds as biomarkers in breath analysis by different analytical techniques *Bioanalysis* **5** 2287–306

Callaway T R, Dowd S E, Edrington T S, Anderson R C, Krueger N, Bauer N, Kononoff P J and Nisbet D J 2010 Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded flx amplicon pyrosequencing *J. Animal Sci.* **88** 3977–83

de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, Osborne D and Ratcliffe N M 2014 A review of the volatiles from the healthy human body *J. Breath Res.* **8** 014001

Deneris E S, Stein R A and Mead J F 1985 Acid-catalyzed formation of isoprene from a mevalonate-derived product using a rat-liver cytosolic fraction *J. Biol. Chem.* **260** 1382–5

Dobbelaar P, Mottram T, Nyabadza C, Hobbs P, ElliottMartin R J and Schukken Y H 1996 Detection of ketosis in dairy cows by analysis of exhaled breath *Vet. Q.* **18** 151–2

Draksler D, Locascio M, Gonzalez S and Oliver G 2002 The development of faecal flora in young creole goats *Small Rumin. Res.* **46** 67–70

Elliott-Martin R J, Mottram T T, Gardner J W, Hobbs P J and Bartlett P N 1997 Preliminary investigation of breath sampling as a monitor of health in dairy cattle *J. Agric. Eng. Res.* **67** 267–75

Ellis C K, Stahl R S, Nol P, Waters W R, Palmer M V, Rhyan J C, VerCauteren K C, McCollum M and Salman M D 2014 A pilot study exploring the use of breath analysis to differentiate healthy cattle from cattle experimentally infected with mycobacterium bovis *PLoS One* **9** e89280

Filipiak W *et al* 2012 Dependence of exhaled breath composition on exogenous factors, smoking habits and exposure to air pollutants *J. Breath Res.* **6** 036008

Garner C E, Smith S, Costello B D, White P, Spencer R, Probert C S J and Ratcliffe N M 2007 Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease *Faseb J.* **21** 1675–88

Guarner F and Malagelada J R 2003 Gut flora in health and disease *Lancet* **361** 512–9

Hales K E, Parker D B and Cole N A 2012 Potential odorous volatile organic compound emissions from feces and urine from cattle fed corn-based diets with wet distillers grains and solubles *Atmos. Environ.* **60** 292–7

Kim M, Kim J, Kuehn L A, Bono J L, Berry E D, Kalchayanand N, Freetly H C, Benson A K and Wells J E 2014 Investigation of bacterial diversity in the feces of cattle fed different diets *J. Animal Sci.* **92** 683–94

Knobloch H, Schroedl W, Turner C, Chambers M and Reinhold P 2010 Electronic nose responses and acute phase proteins correlate in blood using a bovine model of respiratory infection *Sensors Actuators B-Chem.* **144** 81–7

Krüger C, Kohler H and Liebler-Tenorio E M 2015 Sequential development of lesions 3, 6, 9, and 12 months after experimental infection of goat kids with mycobacterium avium subsp paratuberculosis *Vet. Pathol.* **52** 276–90

Kuzma J, Nemecekmarshall M, Pollock W H and Fall R 1995 Bacteria produce the volatile hydrocarbon isoprene *Curr. Microbiol.* **30** 97–103

Lord H L, Zhan W and Pawliszyn J 2010 Fundamentals and applications of needle trap devices: a critical review *Anal. Chim. Acta.* **677** 3–18

McCarthy R D and Kesler E M 1956 Relation between age of calf, blood glucose, blood and rumen levels of volatile fatty acids, and *invitro* cellulose digestion *J. Dairy Sci.* **39** 1280–7

Metzler-Zebeli B U, Schmitz-Esser S, Klevenhusen F, Podstatzky-Lichtenstein L, Wagner M and Zebeli Q 2013 Grain-rich diets differently alter ruminal and colonic abundance of microbial populations and lipopolysaccharide in goats *Anaerobe* **20** 65–73

Miekisch W, Herbig J and Schubert J K 2012 Data interpretation in breath biomarker research: pitfalls and directions *J. Breath Res.* **6** 036007

Miekisch W, Schubert J K and Noeldge-Schomburg G F E 2004 Diagnostic potential of breath analysis—focus on volatile organic compounds *Clin. Chim. Acta* **347** 25–39

Miekisch W, Trefz P, Bergmann A and Schubert J K 2014 Microextraction techniques in breath biomarker analysis *Bioanalysis* **6** 1275–91

Mieth M, Kischkel S, Schubert J K, Hein D and Miekisch W 2009 Multibed needle trap devices for on site sampling and preconcentration of volatile breath biomarkers *Anal. Chem.* **81** 5851–7

Mochalski P, King J, Klieber M, Unterkofler K, Hinterhuber H, Baumann M and Amann A 2013 Blood and breath levels of selected volatile organic compounds in healthy volunteers *Analyst* **138** 2134–45

Peled N, Ionescu R, Nol P, Barash O, McCollum M, VerCauteren K, Koslow M, Stahl R, Rhyan J and Haick H 2012 Detection of volatile organic compounds in cattle naturally infected with mycobacterium bovis *Sensors Actuators* B **171** 588–94

Phillips M, Herrera J, Krishnan S, Zain M, Greenberg J and Cataneo R N 1999 Variation in volatile organic compounds in the breath of normal humans *J. Chromatogr. B, Biomed. Sci. Appl.* **729** 75–88

Piatkowski B, Gürtler H and Voigt J 1990 *Grundzüge der Wiederkäuer-Ernährung* (Jena: Gustav Fischer) pp 35–57

Piccione G, Sciano S, Messina V, Casella S and Zumbo A 2011 Changes in serum total proteins, protein fractions and albumin–globulin ratio during neonatal period in goat kids and their mothers after parturition *Ann. Anim. Sci.* **11** 251–60

Pleil J D 2009 Influence of systems biology response and environmental exposure level on between-subject variability in breath and blood biomarkers *Biomarkers* **14** 560–71

Pleil J D, Stiegel M A and Risby T H 2013 Clinical breath analysis: discriminating between human endogenous compounds and exogenous (environmental) chemical confounders *J. Breath Res.* **7** 017107

Purkhart R, Koehler H, Liebler-Tenorio E, Meyer M, Becher G, Kikowatz A and Reinhold P 2011 Chronic intestinal mycobacteria infection: discrimination via voc analysis in exhaled breath and headspace of feces using differential ion mobility spectrometry *J. Breath Res.* **5** 027103

Schubert R, Schwoebel H, Mau-Moeller A, Behrens M, Fuchs P, Sklorz M, Schubert J K, Bruhn S and Miekisch W 2012 Metabolic monitoring and assessment of anaerobic threshold by means of breath biomarkers *Metabolomics* **8** 1069–80

Schultz L H 1968 Ketosis in dairy cattle *J. Dairy Sci.* **51** 1133–40

Schulz S and Dickschat J S 2007 Bacterial volatiles: the smell of small organisms *Nat. Prod. Rep.* **24** 814–42

Simon G L and Gorbach S L 1984 Intestinal flora in health and disease *Gastroenterology* **86** 174–93

Spanel P and Smith D 2011 Volatile compounds in health and disease *Curr. Opin. Clin. Nutrition. Metab. Care* **14** 455–60

Spinhirne J P, Koziel J A and Chirase N K 2004 Sampling and analysis of volatile organic compounds in bovine breath by solid-phase microextraction and gas chromatography–mass spectrometry *J. Chromatogr.* A **1025** 63–9

Thorn R M and Greenman J 2012 Microbial volatile compounds in health and disease conditions *J. Breath Res.* **6** 024001

Trefz P, Kischkel S, Hein D, James E S, Schubert J K and Miekisch W 2012 Needle trap micro-extraction for voc analysis: effects of packing materials and desorption parameters *J. Chromatogr.* A **1219** 29–38

Trefz P, Koehler H, Klepik K, Moebius P, Reinhold P, Schubert J K and Miekisch W 2013a Volatile emissions from mycobacterium avium subsp. Paratuberculosis mirror bacterial growth and enable distinction of different strains *PLoS One* **8** e76868

Trefz P, Rosner L, Hein D, Schubert J K and Miekisch W 2013b Evaluation of needle trap micro-extraction and automatic alveolar sampling for point-of-care breath analysis *Anal. Bioanal. Chem.* **405** 3105–15

Turner C, Knobloch H, Richards J, Richards P, Mottram T T F, Marlin D and Chambers M A 2012 Development of a device for sampling cattle breath *Biosyst. Eng.* **112** 75–81

van der Vusse G J 2009 Albumin as fatty acid transporter *Drug. Metab. Pharm.* **24** 300–7

Van Soest P J 1994 *Nutritional Ecology of the Ruminant* (Ithaca NY: Cornell University Press) pp 230–52

Varner S L and Breder C V 1981 Headspace sampling and gas chromatographic determination of styrene migration from food-contact polystyrene cups into beverages and food simulants *J. Assoc. Off. Anal. Chem.* **64** 1122–30

Varshney A, Sen P, Ahmad E, Rehan M, Subbarao N and Khan R H 2010 Ligand binding strategies of human serum albumin: how can the cargo be utilized? *Chirality* **22** 77–87

Walton C *et al* 2013 Analysis of volatile organic compounds of bacterial origin in chronic gastrointestinal diseases *Inflamm. Bowel Dis.* **19** 2069–78

Wang Z N and Wang C J 2013 Is breath acetone a biomarker of diabetes? A historical review on breath acetone measurements *J. Breath Res.* **7** 037109

Weimer P J 1992 Cellulose degradation by ruminal microorganisms *Crit. Rev. Biotechnol.* **12** 189–223

Withey J R 1978 The toxicology of styrene monomer and its pharmacokinetics and distribution in the rat *Scand. J. Work Environ. Health* **4** 31–40 suppl 2