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ORIGINAL ARTICLE

Metabolome of canine and human saliva: a non-targeted metabolomics study

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Abstract

Introduction Saliva metabolites are suggested to reflect the health status of an individual in humans. The same could be true with the dog (*Canis lupus familiaris*), an important animal model of human disease, but its saliva metabolome is unknown. As a non-invasive sample, canine saliva could offer a new alternative material for research to reveal molecular mechanisms of different (patho)physiological stages, and for veterinary medicine to monitor dogs' health trajectories.

Objectives To investigate and characterize the metabolite composition of dog and human saliva in a non-targeted manner.

Methods Stimulated saliva was collected from 13 privately-owned dogs and from 14 human individuals. We used a non-targeted ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-qTOF-MS) method to measure metabolite profiles from saliva samples.

Results We identified and classified a total of 211 endogenous and exogenous salivary metabolites. The compounds included amino acids, amino acid derivatives, biogenic amines, nucleic acid subunits, lipids, organic acids, small peptides as well as other metabolites, like metabolic waste molecules and other chemicals. Our results reveal a distinct metabolite profile of dog and human saliva as 25 lipid compounds were identified only in canine saliva and eight dipeptides only in human saliva. In addition, we observed large variation in ion abundance within and between the identified saliva metabolites in dog and human.

Conclusion The results suggest that non-targeted metabolomics approach utilizing UHPLC-qTOF-MS can detect a wide range of small compounds in dog and human saliva with partially overlapping metabolite composition. The identified metabolites indicate that canine saliva is potentially a versatile material for the discovery of biomarkers for dog welfare. However, this profile is not complete, and dog saliva needs to be investigated in the future with other analytical platforms to characterize the whole canine saliva metabolome. Furthermore, the detailed comparison of human and dog saliva composition needs to be conducted with harmonized study design.

Keywords Saliva · Human · Dog · Lipid · Metabolomics · Liquid chromatography · Mass spectrometry

Abbreviations

ala	Alanine
arg	Arginine
DAG	Diacylglycerol
FA	Fatty acid
gln	Glutamine
glu	Glutamic acid
gly	Glycine
his	Histidine
ile	Isoleucine
leu	Leucine

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LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
phe	Phenylalanine
pro	Proline
pyroglu	Pyroglutamic acid
ser	Serine
TAG	Triacylglycerol
thr	Threonine
tyr	Tyrosine
val	Valine

1 Introduction

Human saliva has been studied and characterized extensively in recent years. Most of the saliva is water (over 99%) containing a variety of electrolytes, different kinds of proteins as well as low molecular weight (< 1500 Da of mass) metabolites (Dame et al. 2015; Humphrey and Williamson 2001; Gardner et al. 2020). Mucus, epithelial and blood cells, food remainders and traces of medications or chemical products are also found in saliva (Aps and Martens 2005; Elmongy and Abdel-Rehim 2016). Moreover, biological material such as DNA and bacteria with their metabolites exist in saliva (Cuevas-Cordoba and Santiago-Garcia 2014).

Since saliva is rich in small molecules and given its role as "a mirror of the body", there is a growing interest towards saliva usage as a non-invasive sample material for monitoring health trajectories to aid diagnosis or reveal the molecular mechanisms of disease pathologies. The same applies to domestic dogs which suffer from similar diseases to humans such as metabolic diseases, chronic inflammation, and cancers, manifested as diabetes (O'Kell et al. 2017), inflammatory bowel disease (Minamoto et al. 2015) and leukemia (Breen and Modiano 2008), respectively. Physiological similarity with humans and the large size of the canine have been reasons for the rise of these animals to one of the biomedical models alongside the rodents, for example in the study of genomics (Hytonen and Lohi 2016; van Steenbeek et al. 2016) and behavior (Puurunen et al. 2018). Despite the rising interest in dogs and saliva metabolomics, there is no data available for the canine saliva metabolome.

Humans share the same anatomy and salivary gland structure with dogs, except for dogs' zygomatic glands. The basic functions of saliva, such as lubrication, maintenance of oral homeostasis and dental welfare as well as bactericidal effects against pathogens, resemble each other (Dame et al. 2015; de Sousa-Pereira et al. 2015; Humphrey and Williamson 2001). Moreover, dogs use panting and evaporative cooling as the major function when exposed to heat and/or exercise (Goldberg et al. 1981). Differences between human and canine saliva have been revealed in the comparison of the proteome signature where, for example, cystatins with antimicrobial properties have been recognized in lower levels in the saliva of canines compared to saliva of humans (Sanguansermsri et al. 2018). In addition, different antimicrobial protein family members are identified in human and dog saliva, such as cathelicidin 1, cathelicidin antimicrobial peptide and CRISP1 in dog saliva, whereas cathelicidins were not detected in healthy humans but CRISP3 was (de Sousa-Pereira et al. 2015).

Several studies of the human salivary metabolome link it to various conditions, including oral and breast cancers (Sugimoto et al. 2010), type 2 diabetes (Barnes et al. 2014) and Sjögren's syndrome (Mikkonen et al. 2013). Therefore, also the salivary metabolome of the dog could reflect the metabolic activity of canines' oral cavity and total body. In this study, we compared the metabolome of dog and human saliva utilizing UHPLC-qTOF-MS -based non-targeted metabolomics approach. We aimed to identify a wide range of saliva metabolites to explore the metabolic profiles of both species and their overlap.

2 Materials and methods

2.1 Animals and human participants

Voluntary Finnish dog owners were recruited for the canine saliva donation. The saliva collection was conducted from 13 privately-owned dogs with the owners' written consent and presence. The dogs were healthy referring no disease with one exception (cataract) and were not subjected to any drug treatment according to their owners. The breeds were Belgian Sheepdog (n = 2), Belgian Tervueren (n = 2), Weimaraner (n = 2), Rottweiler (n = 3), Golden Retriever (n = 2) and Flat-Coated Retriever (n = 2). The age of the dogs varied from 1.2 years to 9.3 years. The mean age was 5.5 years and SD 2.5 years. The number of males were 5 and females 8. Two of the female dogs were neutered.

Human saliva samples were collected from 14 healthy, non-smoking females, aged between 30 and 70 years (mean age 53 years, SD 11) who were recruited from the dental education clinic of Kuopio University Hospital. The volunteers had no recent history of systemic diseases or were not taking any medication. Inclusion criteria were healthy subjects, with normal excretion of saliva and no medications. Exclusion criteria were smokers, wearing removable dentures, having systemic diseases or medication, having a treatment history for cancer, or being incapable of communication. Out of all the patients examined, no males met these criteria. At the time of the study, every subject underwent an oral and dental examination performed by a dentist, and

their oral health were good, no gingivitis, missing/broken teeth or caries.

2.2 Collection of saliva samples

Canine saliva samples were collected between 9 to 11 a.m. by the same person at the dog's home. The dogs were fasted and rested 12 h before sampling. Saliva was collected without causing any stress or harm to dogs as follows. Salivation was stimulated with prospect of food, i.e. the dog could see or sniff the treat but was not allowed to eat it. Saliva was collected under the tongue and from the surface of the mucosal lining of lips and cheek straight to 1.5 ml Eppendorf tube. The maximum collection time was four minutes. No contaminations, e.g. hair and blood, were observed in visual inspection. Immediately after sampling, proteins were precipitated, and metabolites extracted with two volumes of acetonitrile mixed with 1 volume of saliva simultaneously mixing gently in vortex and finally at maximum speed 10 s. Samples were kept on ice during shipping and stored in − 20 °C 3–5 days prior to metabolomics analysis.

Human saliva samples were collected at least one hour after eating and drinking between 9 and 11 a.m. Stimulated saliva was collected using the standard technique according to Navazesh (1993) as follows. The saliva flow was stimulated by chewing a paraffin wax (1 g; Orion Diagnostica, Espoo, Finland) for 30 s, followed by the collection of the produced saliva into a glass cup for five minutes. Saliva samples were transported to the laboratory on ice, and then clarified by centrifugation (3000×*g*, 20 min, + 4 °C). The supernatants were stored at − 20 °C for later use.

2.3 Sample preparation

Dog and human saliva samples were thawed on ice. Human saliva samples were precipitated and extracted similarly as dog saliva (200 µL of saliva and 400 µL of acetonitrile). All samples were centrifuged (10,600×*g*, 5 min, + 4 °C), and the supernatants were filtrated through 0.2 µm Acrodisc® Syringe Filters with a PTFE membrane (PALL Corporation, Ann Arpor, MI) prior subjecting to the LC–MS analyses. Quality control (QC) samples were made separately from dog and human samples by mixing aliquots of 30 µl from every dog or human supernatant to one tube. QC mixed sample contained aliquots from every dog and human sample mixed into one tube.

HPLC-grade acetonitrile (VWR Chemicals, Fontenay-sous-Bois, France) was used for sample preparation. LC–MS grade methanol (Riedel-de Haën™, Honeywell, Seelze, Germany), HPLC-grade acetonitrile (VWR Chemicals, Fontenay-sous-Bois, France), LC–MS grade formic acid (Fluka™, Honeywell, Seelze, Germany), ammonium formate (Fluka™, Honeywell, Seelze, Germany) and class 1 ultra-pure water (ELGA Purelab ultra Analytical, UK) were used for mobile phase eluents in RP and HILIC chromatographic separation.

2.4 UHPLC-qTOF-MS analysis

The samples were analyzed by a 1290 LC system coupled to a 6540 UHD accurate-mass qTOF spectrometer (Agilent Technologies, Waldbronn, Karlsruhe, Germany) using electrospray ionization (ESI, Jet Stream) in positive (+) and negative (−) polarity. Separation was performed using reversed phase (RP) chromatography with a Zorbax Eclipse XDB-C18 column (2.1 × 100 mm, 1.8 µm, Agilent Technologies, Palo Alto, CA, USA). The column temperature was 50 °C and flow rate 0.4 ml/min. Mobile phase consisted either water (A) or methanol (B) both with 0.1% (v/v) formic acid. The gradient was as follows: 2% B followed by a gradient to 100% B in 10 min, an isocratic step at 100% B for 4.5 min and 2% B for 2 min. Hydrophilic interaction (HILIC) chromatographic separation was performed on Acquity UPLC® BEH Amide column (2.1 × 100 mm, 1.7 µm, Waters Corporation, Milford, MA). The column temperature was 45 °C and flow rate 0.6 ml/min. Mobile phase consisted of 50% v/v acetonitrile in water (A) or 90% v/v acetonitrile in water (B) both with 20 mM ammonium formate buffer. The gradient was as follows: 100% B for 2.5 min followed by a gradient to 0% B in 10 min and 100% B for 2.5 min. The sample volume of 2.0 µl was injected for each chromatographic run.

The ESI source operated using the following conditions: capillary voltage 3500 V, nozzle voltage 1000 V, fragmentor voltage 100 V, skimmer 45 V, nebulizer pressure 45 psi, drying gas temperature 325 °C and flow 10 l/min and sheath gas temperature 350 °C and flow 11 l/min. Mass data were acquired with scan time of 600 ms over a 50–1600 m/z range. For automatic MS/MS analyses, four ions with the highest intensities were selected for fragmentation from every precursor scan cycle where precursor isolation width was set to 1.3 Da. Selected precursor ions were excluded after two product ion spectra and released after a 0.25-min hold. Precursor scan time either ended at 20,000 counts or after 500 ms, depending on the ion intensity. Product ion scan time was 500 ms. Collision energies were 10 V, 20 V and 40 V. Continuous internal calibration was performed during analyses to assure the desired mass accuracy. The reference ions from infusion solution were m/z 121.05087300 and 922.00979800 for positive mode and m/z 112.985587 and 966.000725 for negative mode. For the quality assurance of the chromatographic and mass spectrometry runs, QC mixed sample were injected at the beginning of the analysis and after every 9 samples. Separate dog QC and human QC samples were analyzed in the beginning of the corresponding analysis to provide the MS data, and used for the automatic data-dependent

MS/MS analyses. The data acquisition was accomplished with MassHunter Acquisition B.05.01 software (Agilent Technologies).

2.5 Non-targeted metabolomics data preprocessing

The LC–MS raw data from four different analytical modes (RP+, RP−, HILIC+, HILIC−) was exported to MassHunter Qualitative Analysis B.07.00 (Agilent Technologies, USA) for feature extraction and peak picking combined with chromatographic alignment across all data files per mode. To remove the redundant and non-specific information considered as background noise, peaks with ion abundance less than 10,000 were excluded from further analysis. The feature files were imported as compound exchange format (.cef-files) into Agilent Mass Profiler Professional software (MPP version 13.1.1, Agilent Technologies) for compound alignment yielding a peak list which was exported to Microsoft Excel 2016. Altogether, 8375 molecular features were collected in the four analytical modes. Out of those, molecular features that were present in at least 50% of the samples in either of the sample groups (5468 features) were considered for metabolite identification. Principal component analysis was performed using SIMCA (version 15, Umetrics).

2.6 Metabolite identification

The putative metabolite identification was performed using an open-source software, MS-DIAL (RIKEN PRIMe). Collected MS/MS data was converted as.abf-files using Analysis Base File Converter program (Reifycs Inc.) and converted files were imported to MS-DIAL (versions 2.66 to 3.12). Public databases, Metlin and MassBank of North America (MoNA), and in-house LC–MS/MSMS standard library were downloaded to MS-DIAL for utilization of retention time, accurate mass, isotope ratio and MS/MS spectrum information for peak and metabolite identification. The built-in MS-DIAL library was utilized for lipid identification. Each matched spectrum was manually inspected. The guidelines from Sumner et al. (2007) were used for ranking metabolite identifications as follows: Compounds in identification level 1 were verified by comparing exact mass, retention time and MS/MS fragmentation spectra with in-house LC–MS/MSMS standard library. Compounds in level 2 were matched with exact mass and MSMS spectra from public databases mentioned above. MassHunter Profinder B.08.00 software (Agilent Technologies) was applied for targeted feature extraction to minimize the appearance of false negative features implemented with the manual inspection and integration of the targeted feature.

3 Results

With the aim to explore salivary metabolite composition in dog and human, we focused on 5468 metabolic features collected with four analytical modes using a non-targeted metabolomics approach. A total of 211 metabolites were identified (Table 1) including both endogenous and exogenous compounds. Among those, 31 metabolites (14.6%) were found only in dog saliva, and 9 metabolites (4.2%) only in human saliva (Fig. 1). The identities of 69 metabolites were verified as level 1 identification (Sumner et al. 2007) whereas 142 metabolites were in identification level 2. Characteristics and reference spectra for all identified metabolites in human and dog saliva are given as supplementary material (S1). The identified metabolites were classified as amino acids, amino acids derivatives, biogenic amines, lipids and carnitines, nucleic acid subunits, organic acids, small peptides, chemicals, and other metabolites.

The major difference between the human and dog saliva metabolites was observed in the lipid group. Dog saliva contained 25 lipids or lipid-like molecules (i.e. carnitines), which were absent in the human saliva, including 11 phosphatidylcholines (PC), 6 phosphatidylethanolamines (PE), 3 lysophosphatidylethanolamines (LPE), 2 lysophosphatidylcholines (LPC), 1 diacylglycerol (DAG) and 2 acylcarnitines. In contrast, small peptides, including mostly dipeptides, were more prevalent in human when compared to canine saliva. Dogs were completely lacking eight of the 34 identified small peptides, and in total, 13 dipeptides had minor ion abundance in the dog saliva.

Both the dog and human saliva contained 15 of the 20 generic amino acids. However, asparagine, cysteine, glycine, methionine and valine were not detected from the saliva of both species. The group of amino acid derivatives included ten metabolites. Among those, gamma glutamylglutamic acid was detected only in humans, and phenylacetylglycine only in canines. Besides amino acids and their derivatives, asymmetric dimethylarginine (ADMA), cadaverine, carnosine, creatinine, histamine, spermidine and taurine were identified as biogenic amines. Those seven metabolites and eight different nucleic acid subunits were detected in both species. Furthermore, canine saliva contained also one unique organic acid which was identified as pyrocatechol sulfate, and four other compounds named quinaldic acid, sphinganine, sphingosine and usnic acid.

The entity of identified metabolites in canine and human saliva indicate partially comprised species-specific metabolic profiles (Table 1). In addition, a large variation in ion abundance within and between the identified saliva metabolites were observed in both species. Inter-individual variation and sample variation is shown with descriptive statistics in the supplementary material (S2). Furthermore,

Table 1 Identified metabolites in dog and human saliva in the non-targeted LC–MS analysis

Compound ID	Level of ID	Dog			Human		
		Min	Median	Max	Min	Median	Max
Amino acids							
alanine	1	11621	37378	60490	13180	22480	40910
arginine	1	<10000	214558	654257	436614	1409422	2045075
aspartic acid	1	<10000	19530	56048	66610	249145	399489
glutamic acid	1	45808	352114	795587	92510	219896	477880
glutamine	1	172434	636094	1025305	85529	149544	318895
histidine	1	18000	509729	1065260	354376	488143	1069618
isoleucine	1	13531	324410	752675	31420	140750	246619
leucine	2	24612	286748	458104	20697	119312	196690
lysine	1	<10000	58618	144231	57367	321507	489095
phenylalanine	1	10952	279868	517735	342100	592727	1013511
proline	1	51145	296961	501040	261167	3878169	11401076
serine	2	10401	131521	203482	<10000	37455	88258
threonine	1	<10000	108277	173863	11676	23103	58526
tryptophan	1	<10000	44538	64061	<10000	<10000	34288
tyrosine	1	<10000	25757	41843	79017	183192	412868
Amino acid derivatives							
1-methyl-histidine	1	<10000	60565	129019	11191	18643	50624
3-methyl-histidine	2	<10000	23990	73519	<10000	<10000	43698
5-aminovaleric acid betaine	1	12428	100529	346778	39681	110597	561505
carnitine	1	167694	1081252	1965566	810251	1485396	5209412
citrulline	1	18649	480638	774749	148079	360197	665101
creatine	1	779746	2402765	4766552	1070832	1599528	2560989
gamma glutamylglutamic acid	2	0	0	0	<10000	23557	101558
glycinebetaine	1	3641697	10717865	21103608	450155	1191462	2241265
ornithine	1	<10000	53746	268798	112063	199787	369461
phenylacetylglycine	2	19069	98686	382722	0	0	0
Biogenic amines							
asymmetric dimethylarginine	2	<10000	93638	192806	12057	19586	34784
cadaverine	1	<10000	<10000	70675	20062	106938	336618
carnosine	1	<10000	77531	345281	<10000	<10000	14069
creatinine	1	<10000	3595432	10086481	<10000	1768218	2807040
histamine	1	<10000	<10000	20555	<10000	27233	213795
spermidine	1	<10000	41291	474831	0	346	1729
taurine	2	11922	112591	221966	33028	62705	83051
Lipids and carnitines							
acetylcarnitine	1	65986	876765	2350521	<10000	192280	544614
acylcarnitine C16:0	1	<10000	70459	131534	0	0	0
acylcarnitine C18:0	1	20847	108243	224379	235	1271	2876
acylcarnitine C18:1	2	12269	83360	151949	0	0	0
azelaic acid	1	55255	142717	327229	37854	45207	56463
DAG 34:1	2	<10000	205691	822295	<10000	<10000	23115
DAG 34:2	2	65044	190939	862855	<10000	<10000	15559
DAG 36:3	2	73002	525301	2066243	<10000	<10000	23245
DAG 36:4	2	27386	236542	1097107	<10000	<10000	20966
DAG 38:4	2	52681	237655	907217	0	0	0
FA 15:0	2	42503	381984	1221547	37879	71890	127786
FA 16:0	2	1904573	2225850	2935780	1741092	1969314	2418832
FA 16:1	2	99647	947405	5787185	84217	278554	769808
FA 17:0	1	83142	124843	303479	68962	84249	158464
FA 17:1	2	24010	114688	419195	14253	34183	68761
FA 18:0	1	1906062	2265398	3147739	1762724	2137965	2902604
FA 18:1	1	496478	1830159	3901249	303215	485606	1395954
FA 18:2	2	122906	697812	1022533	86616	245000	857574
FA 18:3	2	<10000	65541	148335	<10000	16226	73462
FA 20:0	1	14102	46432	65739	19776	31424	55360
FA 20:1	2	29563	163202	441862	13801	17572	37779

Table 1 (continued)

Compound ID	Level of ID	Dog			Human		
		Min	Median	Max	Min	Median	Max
FA 20:2	2	16807	179383	325995	<10000	16496	48413
FA 20:3	2	25837	130995	271927	16123	33537	140139
FA 20:4	2	126220	1012611	1694682	<10000	87885	479090
FA 20:5	2	29051	82854	228736	<10000	11784	41867
FA 21:0	2	<10000	18796	125634	2349	3090	4398
FA 22:0	2	15540	28550	48955	<10000	10423	18567
FA 22:1	1	35515	120940	233114	<10000	<10000	11683
FA 22:2	2	<10000	57428	130210	1079	2239	4427
FA 22:3	2	<10000	43436	173654	1854	3440	8009
FA 22:4	2	17392	101471	180448	<10000	<10000	38537
FA 24:1 (n-9)	1	40284	90624	201122	1357	3363	7059
gamma-butyrobetaine	2	14949	248812	751914	228851	711345	1685899
glycerophosphocholine	1	24233	328573	928629	<10000	<10000	23768
hydroxypalmitic acid	2	77734	193115	445803	<10000	11255	17943
isobutyryl carnitine	2	13086	115881	874317	15871	45861	94737
isovalerylcarnitine	1	12599	141423	341344	29009	62403	402540
leucic acid	2	<10000	25422	41427	26369	59768	105831
LPC 16:0	2	95458	648254	16030733	<10000	<10000	19228
LPC 16:1	2	<10000	54524	138021	0	0	0
LPC 18:0	2	113515	455793	4515596	<10000	<10000	41702
LPC 18:1	2	<10000	373385	2099599	0	0	8504
LPC 18:2	2	23760	215712	639442	673	1492	3643
LPE 16:0	2	14886	57102	335617	1119	2268	7056
LPE 16:1	2	<10000	62830	486427	0	0	0
LPE 18:0	2	113221	315339	529906	<10000	<10000	12287
LPE 18:1	2	44841	229410	516071	1050	3011	5784
LPE 18:2	2	21575	100444	298766	0	0	0
LPE 20:4	2	32446	110101	456484	0	0	0
panthenol	2	<10000	45107	229860	326	1009	7555
PC 32:1 (16:0_16:1)	2	125717	433195	1881012	0	0	0
PC 32:1e (16:0e_16:1)	2	156372	808574	1960968	0	0	0
PC 34:1 (16:0_18:1)	2	<10000	959665	2183143	0	0	0
PC 34:2 (16:0_18:2)	1	527876	1679345	3151504	0	0	0
PC 34:2e (16:0e_18:2)	2	<10000	1311992	2878498	0	0	0
PC 34:3 (16:1_18:2)	2	<10000	132082	1016841	0	0	0
PC 36:2 (18:1_18:1)	2	<10000	1048913	2627064	0	0	0
PC 36:3 (18:1_18:2)	2	<10000	1427006	5155045	0	0	0
PC 36:4	2	<10000	247531	2210703	0	0	0
PC 38:4 (18:0_20:4)	2	<10000	853419	1552674	0	0	0
PC 38:5 (18:1_20:4)	2	<10000	448002	1111031	0	0	0
PC 38:5e (18:1e_20:4)	2	<10000	1129401	3377601	2370	4163	5414
PE 28:0 (13:0_15:0)	2	<10000	119668	317171	0	0	0
PE 30:0 (15:0_15:0)	2	<10000	107950	559716	92	514	1695
PE 32:1 (15:0-17:1)	2	37852	204677	437195	<10000	<10000	14513
PE 32:2 (16:1_16:1)	2	19175	158878	338527	0	0	0
PE 33:2 (15:0_18:2)	2	<10000	104006	234261	0	0	0
PE 34:2 (16:1_18:1)	2	<10000	315200	587195	<10000	<10000	13358
PE 36:2 (18:1_18:1)	2	59997	134283	299542	<10000	<10000	19097
PE 36:2e (18:1e_18:1)	2	51929	93390	246276	<10000	<10000	16125
PE 36:3 (18:1_18:2)	2	141166	230330	330667	0	0	0
PE 36:3e (18:2e_18:1)	2	131863	424557	1203428	6967	10393	18368
PE 36:4 (16:0_20:4)	2	77210	222944	387097	0	0	0
PE 36:4e (16:0e_20:4)	2	205650	378956	912821	<10000	<10000	17742
PE 36:5e (16:1e-20:4)	2	101753	176899	409725	<10000	12316	25173
PE 38:4 (18:0_20:4)	2	155607	393155	748449	2183	4915	11117
PE 38:4e (18:0e_20:4)	2	89687	307156	675664	482	1733	3541
PE 38:5 (18:1_20:4)	2	100884	296574	671320	0	0	0

Table 1 (continued)

Compound ID	Level of ID	Dog			Human		
		Min	Median	Max	Min	Median	Max
PE 38:5e (18:1e_20:4)	2	408052	780956	1966641	<10000	13631	29876
PE 38:6e (18:2e_20:4)	2	267091	877130	1714394	<10000	<10000	13788
propionylcarnitine	1	29989	305075	652985	17116	69733	188291
sebacic acid	2	17955	29244	53705	13812	16413	23916
suberic acid	2	18493	36830	71278	10258	15860	18933
TAG 36:0	2	<10000	46413	325421	<10000	<10000	245965
TAG 38:0	2	<10000	16567	278288	<10000	<10000	72190
Nucleic acid subunits							
2'-deoxy-cytidine	2	11574	121793	664924	<10000	<10000	12687
adenine	1	30862	546767	1140050	25195	182329	736118
adenosine	1	1165584	3535798	5904123	<10000	<10000	71880
cytidine	1	19218	341518	1218546	<10000	12904	208364
cytosine	1	<10000	26965	91078	<10000	13350	113782
guanine	2	<10000	36689	189630	<10000	<10000	14927
inosine	1	14277	433451	867561	<10000	28611	540480
N6-methyl-adenine	2	<10000	13774	50640	17787	50615	123176
Organic acids							
4-guanidinobutanoic acid	1	269089	1392982	6177370	<10000	12942	25562
gamma-aminobutyric acid (GABA)	1	<10000	33837	64474	<10000	58789	576304
indoxyl sulfate	1	<10000	47357	305682	<10000	<10000	12160
lactic acid	2	<10000	122285	225557	<10000	36476	86772
pyrocatechol sulfate	2	113205	1745700	3459574	0	0	0
succinic acid	1	45409	140947	229175	93984	476081	797934
Other metabolites							
1-methylnicotinamide	1	10128	161587	389096	<10000	<10000	14391
2-amino-1-phenylethanol	2	<10000	40291	103424	106355	192183	325172
2-amino-2-methyl-1-propanol	2	<10000	107208	587120	<10000	<10000	38069
3-indoleacetic acid	1	<10000	14938	37641	<10000	39463	175354
4-hydroxybenzaldehyde	1	<10000	29604	186914	<10000	<10000	13761
4-methylpyridine	2	481	2233	8898	<10000	22131	98581
5-aminovaleric acid	1	102880	508819	1371563	1149976	4817223	7408576
allantoin	1	91518	228324	647239	34597	63745	264543
caffeine	1	<10000	<10000	24719	234706	974548	1881117
choline	2	2836825	16398828	27260268	<10000	5041236	8221382
hydroxyphenyllactic acid	1	<10000	47289	118171	16951	59103	99507
kynurenic acid	1	42796	326080	961407	247	746	1533
N-acetylgalactosamine 4-sulfate	2	<10000	56180	126171	<10000	<10000	35013
N-acetylglucosamine	2	<10000	<10000	30449	56943	458389	1675208
N-acetylneuraminic acid	1	<10000	47849	176244	99750	245418	1643564
nicotinic acid	1	<10000	75651	175443	<10000	23384	106513
pantothenic acid	1	<10000	41614	103199	2409	5289	<10000
paraxanthine	1	<10000	<10000	36642	94530	398382	821307
phosphocholine	1	<10000	<10000	91148	<10000	261633	1142184
purine	2	82881	1493615	3907676	<10000	135236	186741
quinaldic acid	2	<10000	34591	195869	0	0	0
riboflavin	1	<10000	24184	66906	442	2112	<10000
sphinganine	1	23812	68181	122139	0	0	0
sphingosine	2	34958	188907	233951	0	0	0
theobromine	1	393	3354	<10000	32249	82651	226896
trigonelline	1	<10000	64859	226093	10243	378399	1645666
urea	2	<10000	<10000	172306	63048	104945	176103
urocanic acid	1	<10000	414615	588379	10900	40058	71136
usnic acid	2	<10000	41140	575241	0	0	0
xanthine	1	<10000	43955	117175	29150	158465	394638
Small peptides							
arg-ile	2	<10000	22196	34949	18331	341025	710052
arg-phe	2	<10000	45418	297897	250431	744952	1458725

Table 1 (continued)

Compound ID	Level of ID	Dog			Human		
		Min	Median	Max	Min	Median	Max
arg-ser	2	109	1955	4214	166259	677600	1155202
gly-pro	2	921	7991	24520	59843	255965	656388
gly-tyr	2	0	0	0	10647	93537	242898
his-glu	2	0	0	0	22420	65996	203852
his-gly	2	110	1394	3470	14162	99402	279463
his-his	2	0	0	0	17697	45681	139994
his-ile/leu	2	<10000	11735	16672	<10000	11516	57709
his-ser	2	0	714	1540	50783	251538	532871
ile-ser	2	223	1249	3180	<10000	34407	83363
leu-leu	2	<10000	16538	44365	65277	367415	1188286
leu-phe	2	<10000	<10000	12514	45017	126515	254275
leu-tyr	2	0	609	2477	<10000	49692	142071
lys-phe	2	523	2711	5132	64608	180533	350198
lys-pro	2	0	0	0	<10000	12059	127461
phe-his	2	109	692	3021	49613	422751	760880
phe-ile/leu	2	892	2475	5046	<10000	12556	129260
phe-ile-arg	2	0	0	0	<10000	29575	177209
phe-phe	2	0	643	2844	19985	27603	66633
phe-tyr	2	<10000	17609	74288	<10000	34889	233467
pro-leu	2	0	0	0	<10000	85454	282915
pyroglu-pro	2	0	1647	5592	<10000	22740	131784
ser-ala	2	3800	8553	13588	<10000	74119	357110
ser-ala-arg	2	0	0	0	27032	236658	473215
ser-gln	2	0	0	0	16908	89025	194865
ser-leu	2	<10000	<10000	13591	<10000	55009	187601
ser-pro	2	<10000	14358	31931	59218	230405	1034273
thr-phe	2	0	313	1132	185038	350604	887569
tyr-arg	2	<10000	<10000	19613	65635	183701	1127841
tyr-gly	2	236	635	1812	50474	98522	256180
tyr-ile/leu	2	0	1734	4178	30825	72468	194831
val-arg	2	<10000	<10000	34841	11757	34742	122268
val-leu	2	<10000	14638	37038	30689	206065	882780
Chemical compounds							
dibutyl adipate	2	45845	106927	655818	47565	64526	172698
diethanolamine	1	18671	63434	135897	<10000	33277	451781
diethylhexyl adipate	2	145957	1520708	7535913	58058	101414	1556448
diisodecyl phthalate	2	99927	277741	1640341	208712	222322	247443
dioctyl phthalate	1	434106	1873771	7066364	254467	269640	308677
dodecyl sulfate	2	145172	1097399	2697976	225102	632151	25249152
dodecylbenzenesulfonic acid	2	287702	873888	1751253	15936	213023	566013
linoleamide	2	11863390	30840156	60906180	23231544	31221685	38207392
myristamide	2	1686252	3648810	9409145	2751050	4606192	5908500
oleamide	2	48130344	91514384	132632448	72842344	85608764	100743984
palmitoleamide	2	5279505	12170688	23845604	9643438	12678565	16119980
pentaerythritol tetrakis(3,5-di-tert-butyl-4-hydroxyhydrocinnamate)	2	34593	76145	213762	52410	76586	156358
phthalic acid mono-2-ethylhexyl ester	2	60347	74071	107216	106038	128498	163136
stearamide	2	3478602	9423142	19570576	7812933	10203003	12323552
triethanolamine	2	<10000	117186	4144601	<10000	29260	223469
tris(hydroxymethyl)aminomethane	2	<10000	175962	348895	<10000	180882	239265

Level of identification (ID) based on Sumner et al. (2007).
Minimum, median and maximum abundances are shown.

Ion abundance: 0 - <10000 | 10000-99999 | 100000-999999 | >1000000
Color

the principal component analysis clearly separated the different species, however large variation was also observed within species (supplementary material S3). Example total ion chromatograms of salivary samples of dog and humans from all four analytical modes are shown in the supplementary material (S4).

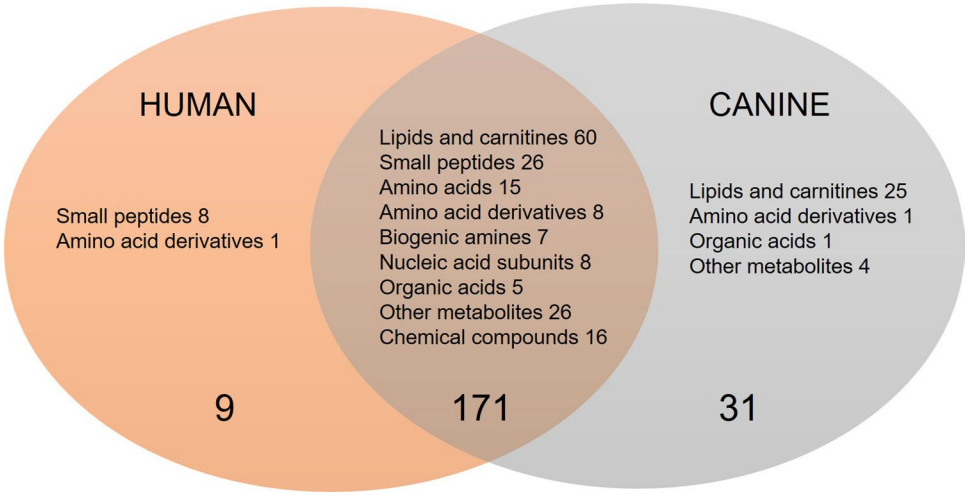

Fig. 1 Venn diagram displaying the shared and unique salivary metabolites by classes among the two species. Metabolite was annotated as unique when the ion abundance was confirmed as zero in all samples per study group. Unique metabolites with zero ion abundance were confirmed with the manual inspection and integration of the targeted feature using MassHunter Profinder B.08.00 software (Agilent Technologies)

4 Discussion

This study provides the first characterization of canine saliva metabolome and compares its content to the human saliva. We identified altogether 211 metabolites in 13 dog and 14 human saliva samples. Dog saliva contained 31 unique metabolites that were mostly lipids and lipid-like molecules. This study demonstrates the feasibility of UHPLC-qTOF-MS in canine and human salivary metabolomics. Exploitation of both, high-resolution precursor and fragmentation data in MS/MS, enables the identification of metabolites that typically exist in lower amounts in saliva than in serum.

Comparison of the content of canine and human saliva revealed differences in the salivary lipids and lipid-like molecules. PCs were not detected in our current analysis in the human saliva, and in addition, eight out of 18 PEs were absent or found with low ion abundance in the human saliva. However, previous studies have reported on PCs in human saliva (Dame et al. 2015). Likewise, our analytical method is capable of detecting PCs, as we have reported them earlier from e.g. human plasma, and therefore most likely other than methodological issues are the reason why they were not detected in the current analysis from human saliva. PCs and PEs are major phospholipids of the plasma membranes in animal cells. This result may indicate the presence of epithelial cell membranes from the oral cavity in the dog saliva samples. Other identified lipids also had lower ion abundance in the human saliva samples when compared with the canine samples. An exception was observed with the most abundant fatty acids in tissues, FA 16:0, FA 18:0 and FA 18:1, which were present with high ion signals also in the human samples. These findings agree with a quantitative study conducted by Larsson et al. (1996) where lipids were detected only in low concentrations in human saliva. Instead, the whole saliva was found to contain more free fatty acids and neutral lipids like di- and triglycerides than polar lipids, such as PCs or PEs (Larsson et al. 1996).

In contrast to lipids, small peptides were found predominantly in human saliva. Canine saliva included only 13 clear signals from di- and tripeptides, whereas 34 were found from human samples. Small peptides in saliva originate from protein degradation induced by host and bacterial proteases (Liebsch et al. 2019). Thus, oral health status, and especially periodontitis, can affect the salivary dipeptides. In the present study, human participants were healthy, and according to dog owners', the dogs were not reported to have any diseases with one exception (cataract). It is unclear if the different fasting time (12 h for dogs versus minimum of one hour for human participants) and/or oral health, combined with the differences in antimicrobial and homeostatic protein compositions between species, affect to peptide levels observed in this study. In addition, the differences in sample collection (e.g. using paraffin wax) and handling may have had an effect.

We identified six additional metabolites, which were present only in the canine saliva. Those included sphingosine and its derivative sphinganine, which are the major bases of the sphingolipids in mammals. Metabolites included also phenylacetylglycine (amino acid derivative) and pyrocatechol sulfate (organic acid) which are reported to be normal human metabolites. Previously, pyrocatechol sulfate was detected in our platform not only in human plasma but also in dog plasma (Hanhineva et al. 2015; Puurunen et al. 2016). Moreover, we putatively identified usnic acid in dog saliva. Usnic acid originates from lichens and might be a trace from a dog food. On the contrary to endogenous metabolites, exogenous compounds in saliva are traces from, for example, food, cosmetics, drug intake and environment (Dame et al. 2015). We identified 16 chemical compounds in both species, including phthalates, which are used as plasticizers. Furthermore, we identified two surfactants, dodecyl

sulfate and dodecylbenzenesulfonic acid, which are used in cosmetics and foods. In addition, fatty acid amides myristamide, palmitoleamide, stearamide, oleamide and linoleamide, were found with high ion abundance in both species. Although these compounds are recognized as endogenous plasma metabolites (Kim et al. 2019), the identified fatty acid amides are also known as lubricants, detergents and softeners which we have found to be derived from the filters used in sample preparation (data not shown). Thus, they are considered as contaminants in this study.

The identified metabolites in dog and human saliva were characterized by inter-individual variation. Several factors, such as diurnal variation, oral health status, physiological condition, gender, age and nutrition are known to have an influence on the metabolite composition of human saliva (Kawanishi et al. 2019; Liebsch et al. 2019; Mikkonen et al. 2013). These factors should be investigated in dogs when the potential of saliva as a sample material for research and diagnostics is discussed as the differences in saliva metabolites between dog breeds, age and sex remain unsolved in this study. Those above-mentioned factors have been identified as affecting the dog plasma metabolome (Lloyd et al. 2016, 2017) and saliva proteome (Pasha et al. 2018). Nevertheless, saliva provides information from several organs and its utility as "the new blood" for the diagnosis and monitoring of human systemic diseases has been studied through omics (Cuevas-Cordoba and Santiago-Garcia 2014). This could also be a case for dogs and veterinary medicine after overcoming the sampling problems and conducting metabolic profiling with larger sample size.

There are some limitations in our study. Firstly, there is no standard operation procedure for collecting dog saliva. In this study, we designed the canine saliva sampling protocol according to the reviewed literature (Elmongy and Abdel-Rehim 2016; Lensen et al. 2015) which aimed to be the most appropriate for the LC–MS analysis. We observed that, even though the dogs were trained for showing their teeth and to cooperate with their owners, the collection of saliva was still tricky to execute due to the characteristic features of saliva being elastic and mucous. Therefore, alternative sampling techniques that are comfortable for the dogs and easy to perform, and which provide enough sample material, need to be developed. Secondly, the non-targeted LC–MS method yields semi-quantitative data suitable for identification and sample-wise comparison but does not provide exact quantities for the detected compounds in saliva. Therefore, when exact quantities are required, other analytical approaches, such as nuclear magnetic resonance spectroscopy (NMR) or targeted LC–MS methods should be applied. However, the low sensitivity of NMR compared to MS limits the detection of the salivary metabolites identified in this study with NMR technique. Furthermore, metabolite identification was focused to the compounds that were classified into identification level 1 and level 2 according Sumner et al. (2007). A wide range of unidentified metabolites still exist in the canine saliva. This is evident according to our data, where in total over 8000 molecular features were detected providing a couple of hundred identifications. The identification of the metabolites behind these molecular features remains a challenge, and only small fraction of measured molecular features can usually be identified in a non-targeted metabolomics study. In addition, the applied LC–MS method does not capture the whole canine saliva metabolome. Characterization of the whole canine saliva metabolome would require use of more diverse set of methods (e.g. NMR and GC–MS) (Dame et al. 2015). Thirdly, our human subjects were all women. For comparing the metabolomes between species, both genders should be included to the study populations despite studies reporting only quantitative differences in salivary metabolites between men and women (Okuma et al. 2017; Takeda et al. 2009). Moreover, more restricted age range could have reduced variation seen in the canine samples. Finally, comparability between human and canine salivary metabolite profiles would improve if more aspects of the study design including sampling protocol could be harmonized between the species.

In conclusion, we were able to identify 211 metabolites in the dog and human saliva using non-targeted metabolite profiling. This study provides novel information that encourages the continuation of the studies with larger cohorts. The results demonstrate the potential of dog saliva metabolome to be used in understanding, for example, disease pathology or changes in metabolism due to xenobiotics or nutrition. Furthermore, saliva could be a source of specific biomarkers also for canines' oral health problems as well as other diseases, but further research is needed to establish and validate the canine saliva biomarkers. Understanding the differences between dogs and humans will then allow the results to be extrapolated to human health.

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Author contributions ST, HL, KH and JP conceived and designed the research of dog saliva. AK designed the research of human saliva. ST conducted canine saliva sample collection and preparation of both canine and human saliva samples. ST performed LC–MS analysis and data analysis with compound identification. OK supervised compound identification. ST drafted the manuscript which was reviewed, edited and approved by all authors.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. OK and KH are founders of Afekta Technologies Ltd., a metabolomics analysis service company.

Ethical approval Human samples: The study was approved by the Research Ethics Committee of the Northern Savo Hospital District (82/2014; 745/2018). The investigation was conducted according to the Declaration of Helsinki. An informed consent to provide saliva for research purposes was given by every donor and a written consent was obtained from every participant. Dog samples: The study was approved by the Finnish national Animal Experiment Board (ESAVI/7482/04.10.07/2015), and the canine saliva samples were collected in accordance with institutional guidelines and in compliance with national and regional legislation. An informed consent to provide canine saliva for research purposes was given by every dog owner and a written consent was obtained from dogs' owners.

Informed consent All authors have approved the version to be published.

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