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Abstract

To expand the knowledge on the porcine salivary proteome, secretions from the three major salivary glands were collected from anaesthetised piglets. Pilocarpine and isoproterenol were simultaneously injected intraperitoneally to increase the volume and protein concentration of the saliva, respectively. The protein composition and relative protein-specific abundance of saliva secreted by the parotid gland and by the mandibular and monostomatic sublingual gland, were determined using iTRAQ. When combining two detection methods, MALDI-TOF/TOF MS and Q-Exactive orbitrap MS/MS, a total of 122 porcine salivary proteins and 6 mammalian salivary proteins with a predicted porcine homolog were identified. Only a quantitative and not a qualitative difference was observed between both ductal secretions. The 128 proteins were detected in both secretions, however, at different levels. Twenty-four proteins (20 porcine and 4 mammalian with a predicted porcine homolog) were predominantly secreted by the parotid gland, such as carbonic anhydrase VI and alpha-amylase. Twenty-nine proteins (all porcine) were predominantly secreted by the mandibular and sublingual glands, for example salivary lipocalin and submaxillary apomucin protein. Data are available via ProteomeXchange with identifier PXD008853.

Significance

In humans, more than 3000 salivary proteins have been identified. To our knowledge, previous studies on porcine saliva only identified a total of 34 proteins. This research increased the total number of identified proteins in porcine saliva to 143. This insight into the porcine salivary proteome will facilitate the search for potential biomarkers that may help in the early detection of pathologies and follow-up of animal welfare. Moreover, it can also endorse the value of a porcine animal model and contribute to a better understanding of the animal's physiology. Additionally, this was the first study to collect and analyse gland specific saliva of pigs. The obtained relative-quantitative knowledge of the identified proteins is valuable when comparing data of stimulated (chewing on a device) vs. unstimulated (passive) saliva collection in the future, since salivary stimulation changes the relative

contribution of the major salivary glands to the whole saliva in the oral cavity. For example, carbonic anhydrase VI, which is present in higher concentrations in parotid saliva, has a higher concentration in stimulated whole saliva because of the larger contribution of the parotid gland after stimulation by chewing.

Keywords

Pig, salivary proteome, gland-specific saliva, iTRAQ, MALDI-TOF/TOF, Q-Exactive orbitrap

1 Introduction

Saliva is an easily accessible body fluid that is widely accepted as a potential medium to assess an individual's health status (e.g. [1]). Using saliva has multiple advantages. It can be collected non-invasively, relatively stress-free and several samples can be taken over a short time period. As a result, saliva collection from challenging populations such as children, disabled or anxious persons and animals could be preferred over blood sampling. In addition, taking a saliva sample only requires limited training, so there is no need for highly trained staff.

Saliva consists for 99% of water, complemented with a wide spectrum of proteins, peptides, hormones, nucleic acids and electrolytes [2-4]. In particular the proteins are investigated as potential salivary biomarkers. The two criteria to be met by a biomarker are (1) the possibility to measure the presence or the abundance of an individual protein, or a set of proteins, and (2) that its presence or abundance indicates normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [5]. In humans, more than 3000 salivary proteins have been identified of which approximately 27% derive from the blood stream through diffusion, filtration or active transport (e.g. [6-8]). This means that not only local pathologies including Sjögren's syndrome [9], oral squamous cell carcinoma [10] or dental caries [11], but also systemic diseases, such as diabetes type 2 [12], lung cancer [13] and cardiovascular pathologies [14], could possibly be detected by a set of salivary biomarkers.

Also in veterinary medicine, interest in salivary biomarkers has tremendously risen during the last decade. In pig production, saliva is already being used to detect specific infections such as porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) (e.g. [15-17]). However, the search for more general biomarkers for infections and non-infectious adverse conditions, such as stress, intensifies. To facilitate this search, knowledge of the porcine salivary proteome is prerequisite. To our knowledge, previous studies on porcine saliva identified a total of 34 proteins (Supplementary file 1) [16, 18-48]. Of these 34 proteins, 21 were identified for the first time using gel-based proteomics [19-21,

48], 12 using immune- or enzymatic assays or techniques relying on antibodies [24, 27, 31, 34, 37, 38, 40, 43, 49], while only one protein was identified using off-gel proteomics [22]. Since this number represents only a fraction of the number identified in human saliva, it is expected that more porcine salivary proteins are to be found. Insight into the porcine salivary proteome would not only facilitate the search for potential biomarkers, it can also endorse the value of a porcine animal model and contribute to a better understanding of the animal's physiology.

To further characterise the proteome of porcine saliva, uncontaminated saliva needs to be collected. However, whole saliva, i.e. saliva that is secreted in the oral cavity and mixed with gingival crevicular fluid, buccal cells and microorganisms is contaminated with a.o. food remnants. To avoid the latter, salivary sampling in humans is always preceded by a fasting period and a rinse of the oral cavity. Since this is difficult to achieve in pigs, uncontaminated saliva can only be collected from anesthetised pigs in the form of gland-specific saliva, more specifically as a ductal secretion, before contamination with a.o. food remnants can occur. In pigs, saliva is mainly produced by three major paired salivary glands, i.e. the parotid gland, the mandibular gland and the sublingual gland. The latter has a monostomatic and a polystomatic compartment. The minor labial, lingual and buccal glands secrete smaller amounts of saliva [50].

The goal of the present study was to expand the knowledge of the porcine salivary proteome by using shotgun proteomics applied to gland-specific saliva. In addition, the relative abundance of the identified proteins was determined by means of an isobaric labelling method combined with complementary tandem mass spectrometry.

2 Materials and methods

2.1 Animals

Four piglets (Belgian Landrace × Piétrain), two animals of either sex, were transported from a local farm to the University of Antwerp at the age of 7 days. They were housed on

commercial brooders (Rescue Decks®, S&R Resources LLC, Mason, USA) and artificially reared on milk formula (BIGGILAC PL+, AVEVE, Antwerp, Belgium), which was provided ad libitum, until the age of 21 days. Piglets had free access to water and were maintained under standard environmental conditions (12h/12h light/dark cycle, temperature adjusted to age). The animals were observed daily to document their general health status (body temperature, body weight, food and water consumption, general behaviour and signs of disease (e.g. diarrhoea) or distress (e.g. apathy)). All experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp, Belgium (2014-01) and were in accordance to the European Directive (2010/63/EU).

2.2 Sample and data collection

The 21-day-old animals were anesthetised by means of an intramuscular injection (0.22 ml/kg) of a mixed solution containing Zoletil 100® (tiletamine 50 mg/ml, zolazepam 50 mg/ml; Virbac, Louvain, Belgium) and Sedaxyl® (xylazine hydrochloride 20 mg/ml; VMD, Arendonk, Belgium). To collect saliva from the parotid gland, a modified Lashley cup [51] was three-dimensionally printed (Materialise HQ, Louvain, Belgium) in stainless steel in order to meet the required dimensions. The central part of the Lashley cup was positioned over the parotid papilla, which is a protrusion of the buccal mucosa located at the level of the third to fourth upper premolar [50]. The outer part was vacuumed to secure the cup against the buccal mucosa. The ipsilateral sublingual caruncle was cannulated using a 26 gauge I.V. catheter to collect mixed saliva originating from both the mandibular and monostomatic sublingual glands. Pilocarpine (2 mg/kg; Sigma Aldrich, Diegem, Belgium), which is a parasympathicomimetic drug (M_3-receptor agonist), and isoproterenol (2 mg/kg; Sigma Aldrich), which is a symphaticomimetic drug (β-receptor agonist), were simultaneously injected intraperitoneally. This dual stimulation is thought to mimic salivary reflex secretion since both the parasympathetic and the sympathetic stimulation of acinar cells are necessary to induce salivary secretion [52]. The gland-specific secretions were collected in iced low-protein binding microcentrifuge tubes (Thermo Scientific, Brussels, Belgium) that were

weighed before and after collection to estimate the collected volume, assuming that the specific gravity of saliva is 1.0 g/cm^3 (e.g. [53, 54]). Additionally, the collection time was recorded to estimate the salivary flow rate. Multiplying the concentration by the flow rate enabled us to determine the protein secretion rate, which could be a useful factor to normalise relative abundance data. All collected samples were immediately stored at -80°C until further analysis.

2.3 Sample preparation for shotgun proteomics

To gain a more detailed insight into the salivary proteome, proteins from the eight salivary samples were labelled using an 8-plex kit of isobaric tags for relative and absolute quantification (iTRAQ) reagents and buffers according to the manufacturer's guidelines (Applied Biosystems Sciex Inc., MA, USA). In brief, appropriate volumes containing 100 µg of protein were taken from the four parotid and the four mandibular/sublingual samples, after determination of the total protein concentration using a bicinchoninic acid assay (BCA, Thermo Scientific). From each of these eight samples, proteins were extracted by means of acetone precipitation to discard any salts or lipids. The obtained protein pellets were resuspended in 500 mM triethylammonium bicarbonate (TEAB). Hydrogen bonds were disrupted and disulphide bonds reduced using 2% sodium dodecyl sulphate (SDS) and 50 mM tris-(2-carboxyethyl) phosphine (TCEP), respectively. To alkylate thiols reversibly, the samples were incubated with 200 mM methyl methanethiosulfonate (MMTS). Subsequently, trypsin (Promega, Leiden, The Netherlands) was added to digest proteins during the overnight incubation step at 37°C. Finally, the eight samples were labelled using the eight different iTRAQ reagents (label 113: piglet 1, mandibular/sublingual saliva; label 114: piglet 1, parotid saliva; label 115: piglet 2, mandibular/sublingual saliva; label 116: piglet 2, label 117: parotid saliva; piglet 3, mandibular/sublingual saliva; label 118: piglet 3, parotid saliva; label 119; piglet 4, mandibular/sublingual saliva; label 121: piglet 4, parotid saliva) and combined, resulting in one sample for further analysis by two-dimensional liquid chromatography combined with tandem mass spectrometry (2D-LC-MS/MS).

2.4 First-dimensional separation

The combined sample was fractionated in a first dimension by strong cation exchange (SCX) chromatography using a Waters Alliance e2695 HPLC system with Photo Diode Array detector (Waters NV/SA, Zellik, Belgium). After acidification to a pH of 2.7, the sample was loaded onto a polysulfoethyl-aspartamide SCX-column (2.1 mm x 200 mm; 5 µm particles; PolyLC Inc., Columbia, MD, USA). Three different solvents (solvent A: 10 mM KH_2PO_4, 20% ACN (pH 2.7); solvent B: 10 mM KH_2PO_4, 650 mM KCl, 20% ACN (pH 2.7) and solvent D: 10 mM KH_2PO_4, 650 mM KCl, 20% ACN (pH 4.7)) were used in order to separate the combined peptide sample according to their charge. First, only solvent A was used for 10 min followed by a salt gradient (7.5 – 30%) of solvent B during 45 min and a pH gradient (30-100%) of solvent D for 15 min, with a final 5 min step of only solvent D to eluate highly charged peptides. During the entire gradient, a flow rate of 200 µl/min was kept constant. In total, ten fractions were collected of which the total peptide concentration was determined using the area under the curve (AUC). These fractions were lyophilised and frozen until further analysis.

2.5 Second-dimensional separation and peptide analysis

2.5.1 Micro-capillary RP-HPLC and MALDI-TOF/TOF analysis

The ten SCX fractions were resuspended in solvent A (95% water, 5% ACN, 0.1% formic acid (FA)) to allow for further fractionation using a micro-capillary HPLC system (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany). A guard column (0.3 mm x 5 mm; particle size 5 µm; Zorbax 300 SB-C18, Agilent Technologies) was connected to a C18 analytical RP column (0.3 mm x 150 mm; particle size 3.5 µm; Zorbax 300 SB-C18, Agilent Technologies). A total of 15 µg, from each of the ten previously generated fractions, was loaded using solvent A followed by an elution with solvent B (10% water, 90% ACN, 0.1% FA) using the capillary pump at a flow rate of 6 µL/min. One technical replicate was performed. The gradient intensified from 5% to 55% in the first 56.7 min and quickly rose to 90% in the subsequent 3.3 min. This fractionation step separated each of the ten fractions

into 350 spots (800 nL/spot) on an Opti-TOF® MALDI-plate (28 columns x 25 rows, 700 spots, 2 samples per plate; Applied Biosystems). Afterwards, a matrix consisting of 2.5 mg/ml α-cyanohydroxycinnamic acid in 70% ACN with an internal calibrant (63 pmol/ml human Glu-1-fibrinopeptide B, m/z 1570.6670) was used to cover the spots.

Spotted C18 fractions were analysed using a matrix assisted laser desorption/ionization (MALDI) AB4800 proteomics analyser (Applied Biosystems). Spots that generated precursors with a signal-to-noise (S/N) ratio above or equal to 100 after MALDI-TOF (MS) analysis (reflectron mode; 25 x 20 laser shots per spot; mass range: 800 – 3000 Da; laser intensity: 3300) were selected for MALDI-TOF/TOF (MS/MS) analysis (25 x 20 laser shots per spot; laser intensity: 4350). A maximum of 50 unique precursors per spot were selected for fragmentation in a collision cell (1 kV collisions (positive mode) with air), starting from the precursor with the lowest S/N-ratio.

2.5.2 Nano-capillary RP-HPLC and Q-Exactive orbitrap MS/MS analysis

The ten SCX fractions that were used for the previous analysis using MALDI-TOF/TOF were desalted by using solid phase extraction (SPE). GracePure™ SPE C18 Columns (W. R. Grace & Co.-Conn., Maryland, USA) were placed onto a vacuum manifold and subsequently conditioned (three times with 100 µl methanol) and equilibrated (twice with 100 µl LC-MS H_2O) before the fractions were loaded (two times, reloading the eluate), washed (twice with 100 µl (20% methanol, 80% LC-MS H_2O)) and eluted (twice with 100 µl (40% methanol, 40% ACN, 20% 0.1% HCl in LC-MS H_2O)). The eluted peptides were subsequently lyophilised and frozen until further analysis. Each SCX fraction was separated in a second dimension by RP-C18 chromatography on an Easy nanoLC system using an Acclaim C18 PepMap®100 column (75 µm x 2 cm, 3 µm particle size) connected to an Acclaim PepMap™ RSLC C18 analytical column (50 µm x 15 cm, 2 µm particle size) (Thermo Scientific). Before loading, the vacuum-dried peptide pellets were dissolved in mobile phase A (2% ACN and 0.1% FA), and spiked with 20 fmol Glu-1-fibrinopeptide B (Glu-fib, Protea biosciences, Morgantown, WV, USA). Of each fraction, 1 µg of peptides was loaded onto the column. One technical replicate

was performed. A linear gradient of mobile phase B (0.1% FA in 95% ACN) from 2% to 45% in 55 min followed by a steep increase to 100% mobile phase B in 5 min was used at a flow rate of 300 nl/min. Liquid chromatography was followed by MS and was performed on a Q-Exactive plus mass spectrometer equipped with a nanospray ion source (Thermo Fisher, Waltham, MA, USA). The high-resolution mass spectrometer was set up in an MS/MS mode in which a full scan spectrum (350 – 1850 m/z, resolution 70,000) was followed by a maximum of five high energy collision activated dissociation (HCD) tandem mass spectra (100 to 2000 m/z). The normalised collision energy was set at 33%. A dynamic exclusion list of 15 s for data dependent acquisition was applied.

2.6 Database searching

Proteome Discoverer 2.1 software (Thermo Scientific) was used to export the acquired MS/MS spectra to Mascot generic files. All MS/MS spectra were analysed by Mascot (version 2.5.1; Matrix Science, London, UK) (Supplementary file 4: raw output Mascot) using the Swiss-Prot database (Mammalia, 2017, 65789 entries) or the *Sus scrofa* database was generated based on characterised porcine proteins and the porcine genome (*Sus scrofa* database (reviewed + unreviewed), generated from Uniprot (2018/01/10), 50045 entries). To be complete, a search using the NCBI database (taxonomy *Sus scrofa* generated from NCBI (2018/01/10), 87942 entries) was additionally performed. Since this database did not lead to the identification of more proteins than the Uniprot *Sus scrofa* database and because the latter database is more curated, only the results of the Uniprot search are presented in this manuscript. Methylmethanethiosulfonate binding to cysteine and iTRAQ 8-plex labelling of lysine and the N-terminus were specified as fixed modifications. Oxidation of methionine and iTRAQ 8-plex labelling of tyrosine were set as variable modifications. Beside these common used variable modifications in salivary research, additional searches have been performed using deamidation (NQ), pyro-glutamic formation (E) and/or possible phosphorylations (STY). These variable modifications only had a minimal influence on the outcome of the protein identification search. Therefore, this additional data is not shown in this manuscript.

Analysis was performed based on trypsin digestions, fragment ion mass tolerance of 0.8 Da, and a precursor tolerance of 200 ppm for MALDI-TOF/TOF-spectra and 8 ppm for Q-Exactive orbitrap-MS/MS-spectra. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [55] partner repository with the dataset identifier PXD008853. To avoid misidentifications due to sample contamination the common Repository of Adventitious Proteins was consulted (cRAP) (http://www.thegpm.org/crap/).

2.7 Data analysis

Scaffold Q+ (version 4.7.5; Proteome Software Inc., Portland, USA) was used to validate MS/MS-based peptide and protein identifications [56]. Peptide identifications were only accepted if they could be established at a probability greater than 95% by the stringent Peptide Prophet algorithm [57] with Scaffold delta-mass correction. This additional selection step reduced the number of peptides identified by Mascot, preserving only peptides with a high confidence. Protein identifications were accepted if they met the same probability criterion and contained at least one Scaffold-selected peptide. Protein probabilities were assigned by the Protein Prophet algorithm [58]. The false discovery rate (FDR) was less than 3% for all Mascot searches. All keratins were removed from the output list. A BLAST analysis was performed on all uncharacterised proteins (BLASTP 2.8.0+, All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects Program, *Sus scrofa* (taxid:9823)) [59]. Additionally, all peptides that led to a protein identification with the mammalian database, but not with the porcine database, were also subjected to a BLAST analysis. Only peptides with a 100% identity and query coverage were preserved. Relative quantification was reported by Scaffold Q+ based upon detected iTRAQ reporter ions during tandem mass spectrometric analyses. Since no absolute qualification is possible using this method, the first sample with iTRAQ label 113 was chosen as a reference label and generally received an abundance of 1 for each protein. All other samples/labels

were compared to this reference. The output is a number that indicates a ratio that is relative to this reference sample.

2.8 Statistical analysis

Mixed models were fitted to identify differences between both ductal secretions for the following parameters: concentration, flow rate, protein secretion rate and obtained secretion ratios of all identified proteins. As a consequence, salivary gland was used as a fixed factor. Since one pig produces two samples, data are not independent from each other. Therefore, this fixed factor was nested within an animal to operate as a random intercept in order to account for the variation between the animals. All data were analysed using JMP® Pro 12 (SAS Institute Inc., North Carolina, USA). A *P*-value smaller than or equal to 0.05 was considered statistically significant.

3 Results

3.1 Concentration and flow rate

Collected saliva from the parotid gland had an average protein concentration of 2.85 ± 0.72 mg/ml with an average flow rate of 8.16 ± 3.80 µl/s, meaning that 14.62 ± 10.14 µg of proteins were secreted per second (Table 1). The mandibular and sublingual glands together secreted saliva with a concentration of 1.67 ± 0.45 mg/ml at a flow rate of 12.70 ± 7.12 µl/s, resulting in a protein secretion rate of 39 ± 27 µg/s. The protein concentration of both gland-specific saliva samples differed significantly (P = 0.031), with parotid saliva displaying a higher concentration. Neither the salivary flow rate (P = 0.303) nor the protein secretion rate (P = 0.143) differed significantly between both saliva samples.

3.2 Qualitative data

3.2.1 Identified proteins

Analysing the samples with MALDI-TOF/TOF led to an identification of 21 proteins when using a mammalian Swiss-Prot database as identification source (Fig. 1, Table 2). Although

the technique allowed for the identification of proteins that had not been characterised in porcine saliva before, this number was unexpectedly low. This might be caused by interspecies homology of some proteins since the search algorithm only assigns a unique sequence from the database to a listed identification, hence an underestimation of identifications by Mascot may have occurred. As stated before by others, using larger protein databases requires a higher number of peptide spectra for unambiguous assignment of proteins [60]. To prevent this, a porcine protein database was assembled. Because only 1424 reviewed porcine proteins were available, also unreviewed porcine proteins were included in the porcine database. This database led to the identification of 41 porcine proteins, confirming the identification of 20 proteins that were detected using the mammalian database and adding 21 new porcine identifications. The identification of 1 mammalian protein, being disintegrin/metalloproteinase domain-containing protein 9, was not confirmed. However, a BLAST analysis using a porcine database with the identified peptides indicated that this mammalian protein has a predicted porcine homolog so it was added to the list of identified proteins as mammalian protein with a predicted porcine homolog. To test whether a more sensitive detection method might be advisable, the samples were analysed again, now using a Q-Exactive orbitrap MS instrument. An additional 86 proteins were identified when the search results of both the mammalian and the porcine databases were combined. In total, 122 highly confident porcine proteins and 6 mammalian proteins with a predicted porcine homolog could be identified combining both techniques and databases. However, 25 proteins were reported as uncharacterised proteins, meaning that these proteins lacked annotation. A BLAST analysis with a larger porcine database was conducted to gain insight into the potential function of these proteins. The amino acid sequence of all uncharacterised proteins aligned with a (predicted) protein in the *Sus scrofa* target database. All proteins had an identity percentage that equalled or exceeded 95. Only three proteins, including Ig kappa light chain V-C region (PLC18), MHC class II antigen and envelope glycoprotein, had a slightly lower identity of 87%, 94% and 78%, respectively. Usually, an identification is only considered to be reliable if at least two significant peptides are identified using Mascot.

However, when using the stringent Peptide and Protein Prophet algorithm by Scaffold only reliable peptide identifications remain and we therefore allowed protein identifications based on one 'Scaffold' peptide. This is in line with the view of others who promote the use of protein inference engines instead of implementing rigid protein inference rules [61]. To give an idea, of all 157 proteins identified based on one significant peptide identification by Mascot, 41 proteins passed the stringent Peptide and Protein Prophet algorithm by Scaffold (Supplementary file 2). Additionally, 21 proteins had more than one significant peptide identifications by Mascot but only 1 peptide was assigned to be reliable by Scaffold (Supplementary file 2 and 4). In total, 62 proteins are identified based on one 'Scaffold' peptide. For 45 of these proteins, this peptide was detected in all samples (Supplementary file 2). For 12 proteins this single peptide was only detected in a part of the samples. Finally, for 5 proteins, no iTRAQ-labelled peptides were identified.

3.2.2 Classification of identified proteins

The obtained porcine salivary proteome was plotted against a gene ontology database to generate an overview of the proteins' functions (Fig. 2). From a total of 101 recognisable genes, 61 could be classified in 7 different molecular functions. The majority of the proteins for which these genes encode had catalytic or binding functions (Fig. 2A). These 101 genes were involved in 12 different biological processes resulting in 126 functional hits of which nearly half were involved in cellular or metabolic processes, while only 0.8% could be linked to immunity (Fig. 2B). Only 72 functional hits were assigned to 7 different cellular compartments. A large number of the salivary proteins could be assigned to the extracellular region. However, proteins that were components of the cell, membranes and organelles, were also identified (Fig. 2C).

3.3 Quantitative data

iTRAQ-analysis revealed that all identified salivary proteins were secreted through both glandular ducts, albeit in different concentrations. Of the 128 identified proteins, 24 (18.8%) proteins (20 porcine and 4 mammalian with a predicted porcine homolog) were

predominantly (*P*-value < 0.05) secreted by the parotid gland, while 29 (22.7%) proteins (all porcine) were predominantly (*P*-value < 0.05) secreted by the mandibular and sublingual glands (Fig. 3, Table 2, Supplementary file 2). However, these obtained values are absolute, meaning that one assumes that these glands produce saliva at the same flow rate and with the same concentration of proteins and therefore equally contribute to the composition of whole saliva present in the oral cavity. Since this is not the case, we opted to normalise the obtained values for each protein with the initial protein secretion rate (µg/sec) of each gland. This normalisation reduced the number of proteins predominantly (*P*-value < 0.05) secreted by the parotid gland to 10 (7.%) (9 porcine proteins and 1 mammalian proteins with a predicted porcine homolog), while only 1 (0.8%) of the identified porcine proteins appeared to be secreted in higher concentrations by the mandibular and sublingual glands (Table 2, Supplementary file 2).

4 Discussion

One of the aims of this study was to extend the list of identified porcine salivary proteins. To be able to analyse saliva that is not contaminated with a.o. food remnants, gland-specific saliva was collected. Since it is very difficult to collect ductal secretions in conscious animals, the use of anesthetised animals is recommended (e.g. [62-65]). Unfortunately, salivary flow is low in resting subjects and can even be absent during anaesthesia [52, 54, 66, 67]. Therefore, dual stimulation using pilocarpine and isoproterenol was applied. Pilocarpine stimulates the salivary flow similar to acetylcholine by binding to the M_3-muscarinic receptors on the acinar cells (e.g. [68]). Simultaneously, noradrenaline, mimicked by isoproterenol, binds β_1-adrenoreceptors on acinar cells, which eventually leads to the release of stored proteins into the secreted saliva (e.g. [68]).

4.1 Concentration and flow rate

In this study, the protein concentration was the highest in the parotid saliva, which is in line with previous findings in rats after dual stimulation with isoproterenol and pilocarpine [64, 69].

Moreover, in humans, the parotid gland consistently secretes the highest concentration of proteins, irrespective of stimulation [70, 71]. In contrast, no difference in flow rates of both ductal secretions was observed in the present study. Previous research in humans and rats, however, showed that the combined flow rate of the mandibular and sublingual glands is consistently higher compared to that of the parotid gland, irrespective of stimulation [52, 69, 71]. Whether this discrepancy is due to the used anaesthetics or the applied stimuli, or whether pigs display no differences in flow rate between the different ductal secretions needs to be further investigated. Pentobarbital is a frequently used, non-volatile anaesthetic for animals that could serve as an alternative to the used anaesthetics (e.g. [64, 72]). On the other hand, direct nerve stimulation could be tested as an alternative for chemical stimulation in the anaesthetised animal (e.g. [73, 74]).

4.2 Qualitative data

4.2.1 Identified proteins

Previous studies on porcine saliva could only identify about 32 proteins, while 122 porcine proteins and 6 mammalian proteins with a predicted porcine homolog were detected in the present study. Of these 128 proteins, 40 porcine proteins were identified by both the MALDI-TOF/TOF MS and the Q-Exactive orbitrap MS-technique. It is not surprising that the more sensitive Q-Exactive orbitrap MS/MS technique was able to identify the vast majority of proteins. Two proteins, of which one porcine and one mammalian protein with a predicted porcine homolog, were only detected with the LC-MALDI-TOF/TOF technique, emphasising the need for different ionisation methods to obtain a maximum number of proteins, as has been reported before [60, 75]. This study was also the first to use a gel-free technique to fractionate porcine salivary proteins instead of 2-DE to perform shotgun proteomics [19-21], which could explain the increased number of protein identifications. Both techniques are well-known to be complementary. However, a disadvantage of the gel-free technique is that information about possible post-translational modifications is harder to obtain [76, 77].

An additional explanation for the identification of a larger number of proteins, compared to previous studies, is the use of gland-specific saliva instead of whole saliva. Proteins that are secreted into the saliva by specific glands become diluted once they mix with each other and other fluids in the oral cavity. Moreover, buccal cells and bacteria are usually removed from whole saliva by means of centrifugation or filtration. This step, which was not performed in the present study, could remove macromolecular aggregates or proteins that are bound to bacteria or mucus [78]. For instance lactoperoxidase, two lysozymes and statherin, which are salivary proteins known to be involved in complex formation, were found in the present but not in previous studies that used whole saliva [79, 80].

The importance of using complementary methods not only applies to the techniques, but also to the protein identification database search. Unfortunately, only a mammalian Swiss-Prot database was initially available for protein identification. When using this multispecies database, the presence of proteins with a highly conserved amino acid sequence could cause an underestimation of the number of identified proteins. The reason for this is that the search algorithm will only assign a unique sequence from the database to a detected peptide. This hypothesis appeared to be confirmed since the newly generated porcine database enabled the identification of 55 additional proteins. However, it should be mentioned that the mammalian database only contained reviewed proteins while the porcine database was composed of both reviewed and non-reviewed proteins. Nevertheless, this database appeared to be incomplete since 6 proteins that were found using the mammalian database were not identified using the porcine database. Expanding and further annotating the porcine database will most probably lead to a higher number of identified proteins in the future. This theory confirms why, despite the large number of good-quality mass spectra (5144 spectra for the experiment using MALDI-TOF/TOF MS and 89045 spectra for the experiment using Q-Exactive Orbitrap) only a limited number of proteins is identified.

To our knowledge, from the 32 proteins that were previously detected, 13 were not present in the list of the 128 proteins generated in the present study. Multiple reasons for this discrepancy are possible. Firstly, this exploratory proteomic study analysed gland-specific

saliva that was derived from the three major salivary glands, while most preceding experiments investigated whole saliva that was collected from the oral cavity. Even though the minor glands only produce 1 to 4% of the total salivary volume, their secretions contain some unique proteins [81]. Additionally, whole saliva includes proteins from gingival crevicular fluid, which piles up in the gingival sulci. Though the formation process of this fluid is still under debate, it is considered to be a serum transudate that originates from the gingival plexus of blood vessels in the gingival corium (e.g. [82, 83]). It is known that a.o. enolase proteins and protein S100-A8 and S100-A9 are present in gingival crevicular fluid of humans, but not in saliva from the major salivary glands [84]. These findings suggest that the previously reported proteins in whole saliva that were not detected in the present study, could originate from either the minor salivary glands or the gingival crevicular fluid.

Secondly, the present study analysed the saliva of 21-day-old piglets, whereas older pigs were the subjects of previous studies. It could be hypothesised that the salivary proteome of 21-day-old pigs is still immature and therefore only contains a limited number or a different profile of proteins. This hypothesis has been confirmed in other species (e.g. [64, 85-87]). On the other hand, some proteins that in humans only appear in saliva at a later stage in life, were already present in our young pigs. Developmental dissimilarities could be the cause of these early life differences between human and porcine salivary proteomes. Indeed, pigs already have teeth when they are born, while babies only start teething at the age of 6 to 7 months [50]. Thirdly most previously identified proteins were identified using gel-separation followed by MS-identification, but targeted approaches, such as ELISA, Western blot, TR-IFMA or enzymatic assays, were also used. It has already been proven that a shotgun proteomics approach sometimes fails to identify proteins that are detectable with a targeted approach and vice versa [88].

4.2.2 Classification of identified proteins

The porcine salivary proteins that were identified in this study cover a wide range of molecular weights from which the distribution (46.1% ≤ 40 kDa, 43.0% between 40 and 120

kDa, 10.9% ≥ 120 kDa) largely corresponds to the salivary proteome of human saliva [6]. The majority of these proteins is involved in binding or catalytic activities, which is in line with previous interpretations of the salivary proteome of e.g. humans, rats, mice, dogs, horses, cattle, goats and sheep [6, 88-91]. Additionally, nearly half of all proteins in these investigated proteomes are involved in either metabolic or cellular processes, as was also observed in this study on piglets. More variation was observed when grouping the salivary proteins according to the cellular localisation, but given the limited size of some salivary proteome datasets, conclusions should be drawn with caution [6, 88-91]. To our knowledge, 81.3% of all identified porcine salivary proteins can also be found in saliva of other species (Supplementary file 2) [6, 24, 88-98], indicating that 24 proteins are newly identified salivary proteins.

4.3 Quantitative data

In contrast to humans, no proteins are exclusively secreted by either the parotid gland or the mandibular and sublingual gland in the piglet, although expression levels can vary (Supplementary file 2) [6, 78]. Therefore, in our study, only a quantitative and not a qualitative difference was observed between both ductal secretions. Information about variation in secretion rate or concentration differences of specific proteins in gland-specific saliva is scarce. *Veerman* and his group [70] found that a.o. amylase and proline-rich proteins are secreted at a higher concentration by the parotid gland in humans, which is also the case in pigs. The importance of this quantitative information lies in the fact that the contribution of each gland changes when the salivary flow is stimulated and therefore changes the composition of saliva present in the oral cavity [52]. In humans, the minor glands contribute only 4% of the total salivary volume that is secreted in rest, while the parotid glands contribute 28% and the mandibular/sublingual glands 68%. When the salivary flow is stimulated by tasting, smelling or chewing food, this ratio shifts, increasing the share of the parotid gland to 53%, while reducing the portions of the mandibular/sublingual glands and the minor glands to 46% and 1%, respectively [52]. This means that e.g. amylase and

basic proline-rich protein will have a higher concentration in whole saliva after stimulation because of the larger contribution of the parotid gland. Therefore, comparing proteomics data from stimulated (chewing on a device) vs. unstimulated collection (passive collection) should be performed with caution. However, it should be mentioned that not only the volumetric contribution of the major salivary glands to whole saliva changes during stimulated secretion. The composition of unstimulated and stimulated gland-specific saliva may also diverge. In humans, stimulation with 2% citric acid influences the protein profile of human mandibular and sublingual saliva, but not that of the parotid saliva [71]. It would be valuable to collect both stimulated and unstimulated gland-specific saliva in order to confirm this trend for pigs. Additionally, it is known that the concentration of some salivary proteins is subjected to a circadian rhythm [33, 99]. However, in humans, salivary flow rate is also subjected to this circadian rhythm. Both whole saliva and parotid saliva show a similar rhythm, but with a different amplitude and acrophase. These differences result in an altered percentage contribution of parotid saliva to whole saliva throughout the day with the largest contribution of 32% at 11 a.m. and the lowest contribution at midnight. Therefore, data collected at different time points should be compared with caution. Surprisingly, flow rate does not show a circadian rhythm when salivary flow is stimulated [100].

One should be careful when interpreting the above-mentioned data that are not normalised. These values are absolute, meaning that one assumes that these glands produce saliva at the same flow rate and with the same protein concentration. Since this is not the case, we opted to normalise the obtained values for each protein with the initial protein secretion rate of each gland. Even though this parameter displays some variation, these normalised values represent a better indication of the protein proportion that each gland contributes to the total protein mixture in whole saliva. Unfortunately, correcting for protein secretion rate is a relatively new concept and is not frequently used [101], so information for comparison is scare. Normalisation of values obtained for o.a. amylase and basic proline-rich proteins levelled out any differences in secretion levels between both ductal secretions. In contrast, for some proteins, such as carbonic anhydrase VI and cadherin-1, normalisation did not

change the fact that the parotid gland contributed the largest quantity of this protein to whole saliva. While some proteins displayed no difference in relative abundance before normalisation, such as protein S100-A12 and cystatin-B, they appear to have a higher contribution to whole saliva through the parotid gland after normalisation for flow rate. As mentioned before, these data were collected under non-physiological conditions. It would be valuable in future studies to collect gland-specific saliva under physiological conditions in order to see whether the anaesthesia and chemical stimulation influences the composition and/or protein secretion rate.

An additional advantage of the used method is that knowledge of inter-individual variation is obtained. Proteins such as serpin family I member 1 and peptidylglycine alpha-amidating monooxygenase display very low variation between animals, bearing in mind that only four animals were used. In contrast, proteins statherin and collagen alpha-1(V) chain precursor, for example, are present in very variable concentrations in our porcine samples.

To further explore the salivary proteome of pigs in the future, alternative detection methods or protocol adaptations could be valuable. An example is treating the samples with peptide ligand libraries–to reduce the risk of highly abundant proteins masking the presence of low abundance proteins during LC-MS analysis [102, 103]. Another intervention that would facilitate protein identification is pre-treating the salivary samples with PGNase [104]. It is known that salivary proteins are heavily glycosylated, and to eliminate interferences of this post-translational modification during sample preparation, salivary proteins could be deglycosylated [105].

5 Conclusions

During the present study 122 porcine proteins and 6 mammalian proteins with a predicted porcine homolog were identified of which 111 had never been detected in porcine saliva before. The functional profile of this salivary proteome is similar to that of other species. iTRAQ analysis detected only a quantitative and not a qualitative difference between both ductal secretions. Consequently, the 128 proteins were detected in both secretions, however

at different levels. This relative-quantitative knowledge of the gland-specific salivary proteome is valuable when comparing data between stimulated (chewing on a device) and unstimulated (passive collection) secretions. It needs to be mentioned that normalisation to the initial protein secretion rate of each gland may alter which gland contributed the largest quantity. Even though this parameter displays some variation, these normalised values represent a better indication of the protein proportion that each gland contributes to the total protein mixture in whole saliva.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Tables

Table 1. Salivary protein concentrations, flow rates and protein secretion rates of the collected gland-specific samples. The gender of each 21-day-old animal is specified. An asterisk (*) indicates statistically significant differences.

Animal	Gender	Gland	Protein concentration (mg/ml)	Flow rate (ml/s)	Protein secretion rate (µg/s)
Piglet 1	Female	Mandibular/sublingual	1.10	0.0061	6.68
Piglet 2	Female	Mandibular/sublingual	1.56	0.0045	7.03
Piglet 3	Male	Mandibular/sublingual	2.14	0.0132	28.18
Piglet 4	Male	Mandibular/sublingual	1.88	0.0088	16.61
Piglet 1	Female	Parotid	2.54	0.0090	22.89
Piglet 2	Female	Parotid	3.74	0.0180	67.34
Piglet 3	Male	Parotid	3.06	0.0193	58.96
Piglet 4	Male	Parotid	2.07	0.0045	9.32
Average mandibular/sublingual gland ± SD			1.67 ± 0.45*	0.0082 ± 0.0038	14.62 ± 10.14
Average parotid gland ± SD			2.85 ± 0.72*	0.0127 ± 0.0071	39.63 ± 27.93

Table 2. List of identified proteins in gland-specific saliva of 21-day-old piglets with their molecular weight (MW). A BLAST analysis was performed on all uncharacterised proteins, which are identifiable by the word BLAST in front of their names. The unique peptides identified using MALDI TOF/TOF and/or Q-Exactive orbitrap MS/MS were analysed using the Peptide and Protein Prophet algorithm with Scaffold delta-mass correction. This additional selection step only preserves the identified unique peptides with a high confidence. Proteins indicated with a '†' were only identified using a mammalian database. All the mammalian peptides that led to a protein identification were also subjected to a BLAST analysis using a more complete porcine database. Only peptides with a 100% identity and query coverage were preserved. The predicted porcine protein names are given behind the mammalian homologue.

#	Protein name	MW (kDa)	Number of unique peptides identified using MALDI-TOF/TOF	Number of unique peptides identified using Q-Exactive orbitrap	Gland by which the protein is predominantly secreted (not normalised to protein secretion rate)	*P*-value	Gland by which the protein is predominantly secreted (normalised to protein secretion rate)	*P*-value
1	Carbonic anhydrase VI (*Sus scrofa*)	36	9	12	Parotid gland	< 0.0001	Parotid gland	0.0445
2	Salivary lipocalin (*Sus scrofa*)	22	8	13	Mandibular/ sublingual gland	0.0026		0.2749
3	Lactoperoxidase (*Sus scrofa*)	80	17	29	Parotid gland	0.0037		0.0874
4	Submaxillary apomucin (*Sus scrofa*)	1184	24	33	Mandibular/ sublingual gland	< 0.0001		0.1444
5	Serum albumin (*Sus scrofa*)	70	11	29		0.1085		0.5493

6	Chromosome 6 open reading frame 58 (*Sus scrofa*)	39	6	7	Parotid gland	0.0034		0.0829
7	Cystatin (*Sus scrofa*)	16	3	9		0.1241		0.1633
8	Coagulation factor V (*Sus scrofa*)	256	8	23		0.2357		0.1099
9	Prolactin induced protein (*Sus scrofa*)	12	3	4		0.1975		0.1853
10	BLAST: BPI fold-containing family A member 2 isoform X2 (*Sus scrofa*)	26	7	6	Parotid gland	0.0027	Parotid gland	0.0348
11	Alpha-amylase (*Sus scrofa*)	56	8	11	Parotid gland	0.0435		0.1138
12	BPI fold-containing family A member 1 (*Sus scrofa*)	26	4	6	Mandibular/ sublingual gland	0.0150		0.2869
13	Long palate lung and nasal epithelium protein 1 (*Sus scrofa*)	52	3	16		0.5029		0.0784
14	Angiotensinogen (*Sus scrofa*)	51	5	14	Parotid gland	0.0423		0.1277
15	BLAST: thrombospondin-1 precursor (*Sus scrofa*)	130	5	15	Parotid gland	0.0490		0.0503
16	Basic proline-rich protein (*Sus scrofa*)	62		2	Parotid gland	0.0016		0.0713
17	Carboxylic ester hydrolase (Fragment) (*Sus scrofa*)	39	3	6	Parotid gland	0.0207		0.0971
18	BLAST: zymogen granule protein 16 homolog B (*Sus scrofa*)	19	5	3		0.0992		0.9980
19	Serotransferrin (*Sus scrofa*)	77	3	9		0.1010		0.7975
20	BLAST: secretoglobin family 1D member 1 precursor (*Sus scrofa*)	12	2	2	Mandibular/ sublingual gland	0.0041		0.1327
21	Cholinesterase (*Sus scrofa*)	52	3	3	Parotid gland	0.0027		0.0524
22	BLAST: vitelline membrane outer layer protein 1 homolog precursor (*Sus scrofa*)	22	4	5	Parotid gland	0.0003		0.0645
23	Lysozyme C-3 (*Sus scrofa*)	17	5	3		0.2129		0.1863

24	BLAST: LOW QUALITY PROTEIN: IgGFc-binding protein (*Sus scrofa*)	265	2	8	Mandibular/ sublingual gland	0.0001		0.9814
25	Calcium-activated chloride channel regulator 1 (*Sus scrofa*)	88	2	9	Mandibular/ sublingual gland	< 0.0001		0.6041
26	Statherin (*Sus scrofa*)	8		1		0.317		0.3088
27	BLAST: double-headed protease inhibitor, submandibular gland-like (*Sus scrofa*)	13	2	2	Mandibular/ sublingual gland	< 0.0001		0.4171
28	BLAST: LOW QUALITY PROTEIN: nucleobindin-2 (*Sus scrofa*)	56	2	9		0.0885		0.1699
29	BLAST: multidrug resistance protein 1 isoform X1 (*Sus scrofa*)	139		1		0.3023		0.9001
30	Fstl1 (*Sus scrofa*)	35	1	4		0.0546		0.0684
31	Beta-2-microglobulin (*Sus scrofa*)	13	2	2		0.2423		0.1470
32	Sulfhydryl oxidase (*Sus scrofa*)	81	2	8		0.2057		0.1495
33	Cysteine rich secretory protein 3 (*Sus scrofa*)	27	2	3	Mandibular/ sublingual gland	0.0125		0.1793
34	Chromosome 16 open reading frame 89 (*Sus scrofa*)	41	1	3	Parotid gland	0.0167		0.0976
35	Mucin 7, secreted (*Sus scrofa*)	35	1	2	Mandibular/ sublingual gland	0.0019		0.2205
36	Alpha-2-glycoprotein 1, zinc-binding (*Sus scrofa*)	36		3		0.2497		0.2385
37	Tachykinin 4 (*Sus scrofa*)	12		3		0.0049		0.1027
38	Stromal cell derived factor 4 (*Sus scrofa*)	41		4		0.0633		0.0962
39	Alpha-1-acid glycoprotein (*Sus scrofa*)	23	3	3		0.2751		0.7296

40	Lectin, galactoside-binding, soluble, 3 binding protein (*Sus scrofa*)	61	1	6		0.2109		0.1278
41	Alpha-1-antitrypsin (*Sus scrofa*)	47		3		0.9583		0.0934
42	BLAST: acidic mammalian chitinase precursor (*Sus scrofa*)	52	1	3	Mandibular/ sublingual gland	0.0010		0.5021
43	Serpin family I member 1 (*Sus scrofa*)	46		3		0.3437		0.1328
44	Tissue inhibitor of metalloproteases-2 (*Sus scrofa*)	25	1	1	Parotid gland	< 0.0001		0.0736
45	Alpha-2-HS-glycoprotein (*Sus scrofa*)	39		3	Mandibular/ sublingual gland	0.0262		0.3736
46	Clusterin (*Sus scrofa*)	52		6		0.0768		0.8086
47	BLAST: cadherin-1 precursor (*Sus scrofa*)	98		2	Parotid gland	0.0035	Parotid gland	0.0371
48	BLAST: deleted in malignant brain tumors 1 protein precursor (*Sus scrofa*)	147	1	1		0.1337		0.4588
49	Inhibitor of carbonic anhydrase (*Sus scrofa*)	78		3		0.7833		0.1392
50	Actin, gamma 1 (*Sus scrofa*)	42		2		0.2965		0.1295
51	RNA exonuclease 1 homolog (*Sus scrofa*)	130		1		0.2943		0.1709
52	Peptidyl-prolyl cis-trans isomerase (*Sus scrofa*)	24		5		0.8271		0.1434
53	BLAST: nucleobindin-1 precursor (*Sus scrofa*)	49		4		0.2963		0.1740
54	Myosin binding protein C, slow type (*Sus scrofa*)	136		1		0.6791		0.1455
55	Serum amyloid A protein (*Sus scrofa*)	15	3					
56	Saposin-B-Val (*Sus scrofa*)	58		3	Mandibular/	0.0005		0.3885

					sublingual gland			
57	Long palate lung and nasal epithelium protein 2 (*Sus scrofa*)	50		2		0.0723		0.6492
58	Alpha-2-macroglobulin (*Sus scrofa*)	167		1		0.0574		0.6115
59	Peptidylglycine alpha-amidating monooxygenase (*Sus scrofa*)	106	1	2	Parotid gland	0.0019		0.0930
60	Apolipoprotein A-I (*Sus scrofa*)	30		2	Mandibular/ sublingual gland	0.0001		0.8329
61	Lipocalin-1 (*Sus scrofa*)	19		1		0.1064		0.2681
62	Polyubiquitin-C (*Sus scrofa*)	60	1	2		0.5676		0.1261
63	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 1 (*Sus scrofa*)	106	1	1	Mandibular/ sublingual gland	0.0445		0.6302
64	Odorant-binding protein (*Sus scrofa*)	18		3	Mandibular/ sublingual gland	0.0410		0.4435
65	Pheromaxein C subunit (*Sus scrofa*)	10		2	Mandibular/ sublingual gland	0.0419	Mandibular/ sublingual gland	0.0474
66	Desmocollin 3 (*Sus scrofa*)	100		1		0.4719		0.1098
67	Chitinase 3 like 2 (*Sus scrofa*)	48		1		0.5950		0.1242
68	Potassium voltage-gated channel subfamily J member 6 (*Sus scrofa*)	48		1				
69	IgG heavy chain (*Sus scrofa*)	50		1		0.0899		0.8832
70	Multiple coagulation factor deficiency 2 (*Sus scrofa*)	24		2		0.0734		0.0996
71	Transthyretin (*Sus scrofa*)	16		2		0.0785		0.7136
72	BLAST: Ig kappa chain V-C region	24		2		0.0811		0.7478

	(PLC18) (fragment) (*Sus scrofa*)							
73	Protein S100-A12 (*Sus scrofa*)	11		2		0.3953	Parotid gland	0.0223
74	Peroxiredoxin 5 (*Sus scrofa*)	17		2	Mandibular/ sublingual gland	0.0117		0.4362
75	Alpha-amylase (*Sus scrofa*)	57		2		0.4547		0.1522
76	SIL1 nucleotide exchange factor (*Sus scrofa*)	51		1		0.5231		0.1533
77	Ankyrin repeat domain 24 (*Sus scrofa*)	117		1	Mandibular/ sublingual gland	0.0139		0.3668
78	Tetraspanin (*Sus scrofa*)	26		1		0.1770		0.3003
79	BLAST: pheromaxein C subunit precursor (*Sus scrofa*)	10		1		0.1491		0.5125
80	Carboxylic ester hydrolase (Fragment) (*Sus scrofa*)	37		1	Parotid gland	0.0327		0.0946
81	Hemoglobin subunit beta (*Sus scrofa*)	16		2		0.3100	Parotid gland	0.0071
82	Ribonuclease 4 (*Sus scrofa*)	17		2		0.1350		0.1661
83	BLAST: LOW QUALITY PROTEIN: serpin A3-8 (*Sus scrofa*)	46		1		0.0579		0.7671
84	Cystatin-B (*Sus scrofa*)	11		1		0.6146	Parotid gland	0.0328
85	Cathepsin B (*Sus scrofa*)	37		1	Mandibular/ sublingual gland	0.0021		0.9881
86	Peptidoglycan-recognition protein (*Sus scrofa*)	24		1	Mandibular/ sublingual gland	0.0445		0.2895
87	Insulin-like growth factor-binding protein 4 (*Sus scrofa*)	28		1		0.5712		0.0942
88	O-acyltransferase (*Sus scrofa*)	61		1				

89	BLAST: collagen alpha-1(V) chain precursor (*Sus scrofa*)	184		1		0.6123		0.7351
90	BLAST: chitinase domain-containing protein 1 precursor (*Sus scrofa*)	45		1		0.2409		0.1268
91	Allograft inflammatory factor 1 like (*Sus scrofa*)	16		1				
92	Testis specific serine kinase substrate (*Sus scrofa*)	64		1				
93	BLAST: LOW QUALITY PROTEIN: myosin-10 (*Sus scrofa*)	26		1	Mandibular/ sublingual gland	< 0.0001		0.2346
94	alpha-1,2-Mannosidase (*Sus scrofa*)	73		1	Parotid gland	0.0009	Parotid gland	0.0452
95	Angiomotin (*Sus scrofa*)	118		1	Mandibular/ sublingual gland	0.0163		0.5369
96	BLAST: interleukin-15 receptor subunit alpha isoform X2 (*Sus scrofa*)	18		1		0.2861		0.1176
97	Lysozyme C-1 (*Sus scrofa*)	15		1		0.1854		0.5069
98	Elongation factor 1-alpha (*Sus scrofa*)	50		1	Mandibular/ sublingual gland	0.0054		0.4159
99	B-cell CLL/lymphoma 9 protein (*Sus scrofa*)	149		1		0.9861		0.2702
100	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*Sus scrofa*)	80		1	Parotid gland	0.0172		0.0577
101	Dystroglycan (*Sus scrofa*)	95		1	Parotid gland	0.0120		0.0865
102	Ariadne RBR E3 ubiquitin protein ligase 1 (*Sus scrofa*)	64		1	Mandibular/ sublingual gland	0.1160		0.3289
103	Syncoilin, intermediate filament protein	55		1	Mandibular/	0.0038		0.1321

	(*Sus scrofa*)				sublingual gland			
104	Son of sevenless-like 1 (*Sus scrofa*)	152		1		0.1792		0.1137
105	Sorting nexin 13 (*Sus scrofa*)	98		1		0.2861		0.1782
106	CutA divalent cation tolerance homolog (*Sus scrofa*)	22		1		0.2277		0.2313
107	Cell growth regulator with EF-hand domain 1 (*Sus scrofa*)	26		1	Mandibular/ sublingual gland	0.0115		0.5943
108	BRICHOS domain containing 5 (*Sus scrofa*)	26		1		0.2418		0.1378
109	Heat shock protein HSP 90-alpha (*Sus scrofa*)	85		1	Mandibular/ sublingual gland	0.0092		0.6126
110	BLAST: envelope glycoprotein, partial (*Sus scrofa*)	32		1		0.1327		0.3865
111	Uncharacterized protein (*Sus scrofa*)	9		1		0.4493	Parotid gland	0.0118
112	Ribonuclease T2 (*Sus scrofa*)	23		1		0.3783		0.1606
113	Solute carrier family 38 member 10 (*Sus scrofa*)	111		1		0.4536		0.1858
114	Peptidyl-prolyl cis-trans isomerase A (*Sus scrofa*)	18		1		0.1367		0.1856
115	Sphingomyelin phosphodiesterase acid like 3B (*Sus scrofa*)	45		1	Mandibular/ sublingual gland	0.0363		0.4969
116	Alpha-1-antichymotrypsin 3 (fragment) (*Sus scrofa*)	23		1	Mandibular/ sublingual gland	0.0419		0.3825
117	BLAST: MHC class II antigen, partial (*Sus scrofa*)	12		1	Parotid gland	0.0002	Parotid gland	0.0276

118	60S ribosomal protein L3 (*Sus scrofa*)	46		1		0.4286		0.1857
119	BLAST: heat shock cognate 71 kDa protein (*Sus scrofa*)	71		1		0.3749		0.0920
120	Proteasome 26S subunit, non-ATPase 7 (*Sus scrofa*)	28		1		0.0839		0.2144
121	BLAST: heat shock protein HSP 90-beta (*Sus scrofa*)	83		1		0.1906		0.1520
122	Protease, serine 22 (*Sus scrofa*)	36		1		0.3704		0.0745
123	Formin-2 (*Mus musculus*)† - PREDICTED: Formin-2 (*Sus scrofa*)	167		2	Parotid gland	0.0057		0.0868
124	Methylcytosine dioxygenase TET2 (*Homo sapiens*)† - PREDICTED: methylcytosine dioxygenase TET2-like (*Sus scrofa*)	223		1	Parotid gland	0.0017	Parotid gland	0.0419
125	Alanine--tRNA ligase, cytoplasmic (*Rattus norvegicus*)† - PREDICTED: alanine--tRNA ligase, cytoplasmic (*Sus scrofa*)	106		1	Parotid gland	0.0319		0.0851
126	Sterol O-acyltransferase 2 (*Homo sapiens*)† - PREDICTED: LOW QUALITY PROTEIN: sterol O-acyltransferase 2 (*Sus scrofa*)	59		1				
127	Disintegrin and metalloproteinase domain-containing protein 9 (*Homo sapiens*)† - PREDICTED: disintegrin and metalloproteinase domain-containing protein 9 (*Sus scrofa*)	90	1			0.4119		0.7649

128	Complement C1q tumor necrosis factor-related protein 3 (Homo sapiens)[†] - PREDICED: complement C1q tumor necrosis factor-related protein 3 precursor (*Sus scrofa*)	27		1	Parotid gland	0.0021	Parotid gland	0.0611

Figure captions

Figure 1. Venn diagram of identified proteins combining two analytical methods (MALDI TOF/TOF and Q-Exactive orbitrap MS/MS) and two databases (mammalian and porcine database).

Figure 2. Functional categorisation of the identified porcine salivary proteins, according to their molecular function (A), the biological process in which they are involved (B) or their cellular localisation (C).

Figure 3. Overview of proteins that are present in significant higher concentrations in saliva secreted by either the parotid gland or the mandibular and sublingual gland of 21-day-old piglets. Results are the averages of the relative abundance of each protein ± SD. Proteins indicated with an asterisk are mammalian proteins with a porcine homolog.