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Gender influence on the salivary protein profile of finishing pigs

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ABSTRACT

A study on gender differences in the normal range of biomarkers in porcine saliva samples has the scope for further attention. In the present study, the salivary protein profiles of age-matched healthy male and female finishing pigs were compared. The levels of salivary adenosine deaminase (ADA) activity, haptoglobin (Hp) and C-reactive protein (CRP) have been quantified in 32 male and 32 female pigs to ensure the presence of gender effect on the median levels of salivary biomarkers. Moreover, the total salivary protein content was quantified and compared. The overall salivary protein distribution was compared with SDS-PAGE in 14 male and 14 female pigs and the possible gender influence in the salivary protein profile was analysed by 2DE in 6 male and 6 female pigs. Statistically significant differences were observed in the median values of Hp, CRP, and ADA between male and female pigs ($p < 0.005$). Although the total salivary protein content was not different between the sexes, the salivary protein distribution and profile showed specific gender differences in three proteins of the lipocalin family: the odorant-binding protein, salivary lipocalin and lipocalin 1. These proteins have been related to animal immune status and should be further explored as possible porcine salivary biomarkers.

Significance: The biological relevance of the reported research is based on the possible gender influence on the discovery of salivary biomarkers in porcine production. As differences have been reported in the salivary protein distribution in male pigs in comparison to that of female pigs, the normal-range values, according to gender, of the newly discovered biomarkers should be explored and defined prior to its application in the porcine production system. A hormonal sexual influence is highly hypothesized.

1. Introduction

Gender influence, which has been widely reported in porcine production, is varied from its effects on the performance and dietary feed intake values, with better parameters in sows than barrows [1], to differences in the levels of several biomarkers of health status. As an example, the normal-range levels of acute phase proteins (APP) have been reported to be higher in females than males in the serum [2,3] and saliva samples [4]. Moreover, the effects of gender have been postulated for a long time on the stress markers such as salivary cortisol [5], with higher average basal concentrations in barrows in comparison to gilts. However, no gender difference has been reported in other stress biomarkers, such as Chromogranin A [6].

The importance of gender annotations in research studies is widely discussed in order to provide homogeneous data or to reduce any possible gender effect. Although, the explanations about gender influence on the levels of salivary biomarkers or on the protein distribution in porcine saliva have been studied less.

Unlike the relative stability of genome, the proteome is a dynamic entity that is affected by environmental, genetic, and epigenetic factors. Therefore, in order to catalogue the range of proteins in a specific tissue or biological fluid, it is desirable to analyse the variations caused by sexually dimorphic gene expression (male/female), as it has been reported in porcine muscle proteins [7]. Moreover, it has been published that 5.6% of liver proteins are differentially expressed between the males and females when the liver proteome was analysed by iTRAQ [8]. Taking into account that saliva is composed of a mixture of fluids from different origins - oral fluids, expectorated secretions, serum and blood derivates, bacteria, fungi and viruses and food debris [9] - differences in the salivary protein profile should be also expected.

The saliva is a body fluid that the field of swine herd health management is increasingly interested in. The oral fluid testing was postulated as the optimal sample collection procedure to overcome the shortage of timely information on the circulation of pathogens, since it

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offers an opportunity to easily collect group-level disease data [10]. Moreover, it has been postulated that the measurement of several salivary biomarkers in individual saliva samples has high diagnostic value for disease detection [11]. Thus, detailed information of porcine saliva at the molecular level, including gene and proteome composition, seems to be of great value.

The salivary proteomic analyses reported until now in pigs have been performed exclusively in males, avoiding the possible gender influence on the analysis. However, no evidence about the possible variation of salivary protein profiles in female pigs in comparison to their male counterparts has been published. The present study is focused on the analysis of possible gender differences in the salivary protein profiles of pigs at the last stage of the production system. The overall salivary protein distribution and profile have been compared in age-matched male and female pigs using one- and two- dimensional gel electrophoresis. Moreover, the proteins that appeared differentially regulated in one gender have been identified. The study could serve as a basic data for future studies on salivary proteomics.

2. Material and methods

2.1. Sample size estimation and power analysis

The sample size of the study has been estimated, according to the guidelines of the Ethical Committee for Animal Research in the University of Murcia, using specific statistical tools (http://bit.ly/2eco822). For an expected mean statistical power of 80% and an alpha level of 0.05, the analysis suggested a sample size of 29 animals/group. Taking into account any possible experimental contingencies, an extra 10% has been added, making the total sample size of 64 animals (32 animals/group).

2.2. Animal characterization

A total of 64 Duroc × Large White commercial pigs, from the same farm located in the southeast of Spain, were included in the study. The inclusion criteria were as follows:

- Pigs should be at the last stage of the finishing process.
- Animals should belong to animal-units with similar ages.
- Randomly selection of animals until 32 male and 32 female animals were sampled.
- All animals with any abnormal behaviour or any clinical sign of disease after veterinary inspection were excluded.

The animals were given ad libitum access to a nutritionally balanced commercial diet (3300 kcal ED/kg, 3.1% fibre, 16% protein, and 5% fat). Water was constantly available.

The pigs were housed in unit-pens in groups of 10 with a minimum of 0.65 square meters per animal (Directive 2001/88/CE). Each unit was composed of 10 pens. All the procedures involving animals were approved by the Murcia University Ethics Committee and followed the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe, ETS no. 123). All the methods were performed in accordance with the relevant ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and regulations.

All animals were subjected to a general clinical veterinary examination at the farm. The parameters taken into account during the examination includes the detailed observation of the patient pig and the other pigs in the group and their environment, the general aspect of the individual animals and the annotation of any clinical sign of disease or abnormal behaviour. If any alteration was observed in a pig, it was directly excluded from the analysis. Only one male pig was excluded in the experimental phase of the study, since a mild rectal prolapse was observed during its physical examination.

2.3. Sampling procedure

The saliva samples were obtained prior to the veterinary clinical examination by introducing a sponge, of approximately 1 cm × 1 cm in size, clipped to a thin metal rod, in the mouth of the pigs individually for 1–2 min. The sponges were placed in specific tubes (Salivette tubes, Sarstedt, Nümbrecht, Germany) and stored on ice until processing at the laboratory, for not > 2 h. The saliva was processed by centrifugation of the tubes at 3000 × *g* for 10 min at the laboratory, and was stored in 2 identical aliquots, for biomarker quantification and proteomics analysis respectively, at − 80 °C until analysis.

Two experienced veterinarians collected the saliva samples from all the animals at 2 consecutive days, at approximately 10:00 h a.m., in the beginning of February. The average ambient temperature in the finishing porcine unit at the time of sampling was 19.08 °C.

2.4. Salivary protein measurements

The salivary levels of two acute phase proteins, Hp and CRP, were quantified in all the saliva samples for two purposes. First, to obtain an overall objective inflammatory-infection condition assessment and to identify any possible subclinical disorder, and second, to ensure the gender effect observed in previous studies [11]. For Hp and CRP quantification, previously validated homemade time-resolved immunofluorometric assays were used [4].

In addition, the activity of ADA in the saliva samples was quantified as a novel biomarker of immune status, by using an adaptation of a commercially available enzymatic assay, as recently published [12]. Moreover, the total salivary protein content of all the samples was determined according to Bradford [13].

To test if the values came from a Gaussian distribution, the Kolmogorov-Smirnov normality test was performed for each parameter quantified. As the results did not meet the normal distribution criteria, the median values obtained in males and females were compared with a non-parametric test, the Mann-Whitney test, with a specific statistical software (Graph Pad Prism 5, Graph Pad Software Inc. La Jolla, United States).

The spearmen correlation coefficients were calculated among Hp, CRP, ADA and the total protein measurements in the saliva samples.

2.5. Salivary protein distribution analysis

Afterwards, the saliva samples from 14 male and 14 female pigs were randomly selected and subjected to the SDS-PAGE analysis according to the method of Laemmli [14]. Briefly, 5 μg of the total saliva samples was reduced with dithiothreitol (DTT) at 95 °C and separated with 140 × 140 × 1.5 mm gradient gels (10–15% T, 2.7% C) in a vertical electrophoresis chamber (GE Healthcare, Life Sciences, Munich, Germany). The gels were silver-stained, to visualise the overall protein distribution [15], digitalized with an ImageScanner II (GE Healthcare Life Sciences, Uppsala, Sweden) and analysed by using a specific software (Image Quant TL, GE Healthcare Life Sciences, Uppsala, Sweden). The relative % volume of the different bands in the gel was used for protein distribution comparison as the normalized value. To evaluate the band % volume differences between the sexes, a non-parametric Mann-Whitney test was used with specific statistical software (Graph Pad Prism 5, Graph Pad Software Inc. La Jolla, United States) since the results did not meet the normal distribution criteria after the Kolmogorov-Smirnov normality test.

2.6. 2-DE analysis

The saliva samples from 6 male and 6 female pigs were randomly selected from those subjected to the SDS-PAGE to perform a 2DE analysis. From the selected samples, 30 μg of the total freeze-dried saliva proteins were dissolved in rehydration buffer (8M Urea, 2% CHAPS,