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Transcriptomic signature related to poor welfare of sport horses

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ABSTRACT

The improvement of horse welfare through housing conditions has become a real issue in recent years and have highlighted the detrimental effect of individual housing of horses on their health and behaviour. In this new study, we analysed the blood transcriptome of 45 sport horses housed individually that were previously examined for their behaviour and gut microbiota. We performed differential and regression analyses of gene expression, followed by downstream bioinformatic analyses, to unveil the molecular pathways related to the behavioural changes associated with welfare impairment in these sport horses. We found that aggressiveness towards humans was the behavioural indicator the most correlated to blood gene expression and that the pathways involved belonged mainly to systemic inflammation. In contrast, the correlations between genes, alert postures and unresponsiveness towards the environment were weak. When blood gene expression profiling was combined with faecal microbiota of a sub-population of horses, stereotypies came out as the most correlated to blood gene expression. This study shows that aggressiveness towards humans and stereotypies are behavioural indicators that covary with physiological alterations. Further studies are needed regarding the biological correlates of unresponsiveness to the environment and alert postures.

1. Introduction

The improvement of horse welfare through housing conditions has become a real issue in recent years [1–4]. These recent studies, in addition to others published over the last 20 years, have highlighted the detrimental effect of individual housing of horses on their health and behaviour, mainly due to restrictions regarding social interactions [5], movements [6] and continuous grazing [7]. All three have been shown to be natural needs in a recent meta-analysis [8].

Because welfare is a multidimensional concept including the interaction between physical and psychological components, multidisciplinary approaches are required to give the best possible account of the welfare state of animals [9]. In particular high-throughput molecular technologies provide biological signature of the effects of negative or positive environmental conditions and may help to decipher the contributions of different physiological systems [10]. Although such “omics” technologies have been used to characterise stress responses in various farm animal species such as pig (eg Refs. [11,12]), chicken (eg Refs. [13,14]) or dairy cattle (eg Refs. [15,16]), their use in horse for welfare-related traits are scarce. Along these lines, we and others have recently used gut microbiota profiling [17–20] and blood transcriptomic signatures [21–23] or integrative genomics [24] to complement behavioural indicators of poor welfare in horse.

In this new study, we analysed the blood transcriptome of horses housed individually that were first examined for their behaviour and gut microbiota [4,19]. The study population consisted of sport horses observed for 50 non-consecutive days over nine months. In particular, four behavioural indicators arising from inappropriate living conditions and reflecting poor mental and physical states were studied in depth [4,19,25]. Stereotypies, defined as repetitive unvarying behaviours without obvious goal or function [26] have been related to chronic mental distress [27], immune systems dysfunctions [28] and heath impairments [29]. Aggressiveness towards humans is also related to behavioural restrictions [30] and numerous physiological and health impairments [31,32]. Unresponsiveness to the environment is thought to reflect a depressive-like state [33] in horses and has been related to a

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drop in plasma cortisol, anhedonia [34] and impaired selective attention [35]. Alert postures, reflected by the increase in vigilant behaviours and excessive scanning of the environment [36] could be associated with a general loss of condition [37] and the experience of anxiety. We recently shown that these four behavioural indicators are expressed independently from each other [25] and that they must be assessed individually to evaluate the horses' welfare.

To complete the behavioural investigations of our previous studies and identify physiological covariates that could provide a better understanding of the links between mental and physical health, we performed differential and regression analyses of gene expression to unveil the molecular pathways related to the behavioural changes associated with welfare impairment in sport horses. We found that aggressiveness towards humans was the behavioural indicator the most correlated to blood gene expression and that the pathways involved belonged mainly to systemic inflammation. When blood gene expression profiling was combined with faecal microbiota of a sub-population of horses, blood biochemical and behavioural data, stereotypies came out as the most correlated to blood gene expression and hematocrit:hemoglobin ratio, percentage of monocytes and various bacteria taxa. Altogether, these biological profiling complete the characterisation of several behavioural indicators of poor welfare in horses by revealing their biological correlates.

2. Material and methods

2.1. Animals

The study included 45 sport horses housed in individual boxes in four distinct barns within the same stable, without access to paddocks or pastures, selected among a larger study including 187 horses and described in Ref. [4]. The horses selected were those which showed the most contrasting values for each behavioural indicator. Their individual and housing parameters are presented in Supplementary Table S1. As age, bedding material and the presence of a window in the box opening toward the external environment have been shown to have a significant influence on several behavioural indicators [4], they were included in the analysis.

2.2. Behavioural assessment using the scan sampling method

Each horse was observed on 50 non-consecutive days distributed over nine months, over 90-min observation sessions, ensuring that the sessions were equally distributed across the time of the day (09:00 to 10:30 h, 10:30 to 12:00 h, 12:00 to 13:30 h, 13:30 to 15:00 h and 15:00 to 16:30 h). Each session was repeated ten times. The behavioural assessment was carried out using the scan sampling method [38]: the observer regularly walked in front of the loose boxes at a distance of at least 1.5 m from the door, making as little noise as possible, and recorded the instantaneous activity of each animal (feeding, locomotion, exploration, resting, observation). As this study focused on four behavioural indicators of poor welfare (stereotypies, aggressive behaviours towards humans, the "withdrawn" posture reflecting unresponsiveness to the environment and the alert posture potentially indicating hypervigilance when repeated; Supplementary Table S2), the observer stopped for 5 s in front of the horse if he was expressing one of these indicators, to make sure that it was the right behaviour. For example, the "withdrawn posture" has similar aspects to a standing resting posture and it is necessary to observe the horse for a few seconds to accurately make the distinction. Five scans were recorded per session and the average number of total scans analysed per subject was 195.1 (±22.5) (variations in the number of scans resulted from the absence of the horse or the presence of the caretaker in the loose box at the time of the observation). The frequencies of each of the four behavioural indicators were calculated from the total number of scans recorded per horse.

2.3. Blood RNA extraction

As we wanted to correlate gene and behavioural indicators expression, at first, we selected 32 horses that were the most contrasted for a global welfare score combining the scores obtained for each of the four behavioural indicators four months after the beginning of the study. This date was chosen because it corresponds to a rest period for the horses as there was no competition at the time. The global score for each horse was calculated as below:

$$\text{global welfare score} = (\text{ST} + \text{AG} + \text{UN} + \text{AL}) \ / \ 4$$

where ST, AG, UN and AL are the z-scores for stereotypies, aggressiveness towards humans, unresponsiveness to the environment, and alert postures respectively. The *z*-score for each horse and each behavioural indicator was calculated as below:

$$z - score = \frac{x - \mu}{\sigma}$$

where x is the behavioural indicator value, μ is the behavioural indicator mean for the population and σ is the standard deviation for the population.

The results of this first approach suggested that the behavioural indicators were independent (see Results section). Thus, we decided to performed analyses considering the scores of each behavioural indicator separately. At that end, we used the same cohort to select extreme horses for each behavioural indicator. For aggressiveness towards humans, 2 contrasted groups of 8 horses with high and low scores were selected; for alert posture, 9 horses per group, and for unresponsiveness to the environment, 8 horses per group. As there were only three horses with a high level of stereotypies, we no longer considered this behavioural indicator in the study. Based on this extreme phenotype selection, 45 horses were chosen.

As Ref. [4] showed that some physiological and/or housing factors have a significant influence on behavioural indicators (bedding material and presence in the box of a window opening on the external environment for aggressiveness towards humans, age for unresponsiveness to the environment and bedding material for alert postures), we selected the contrasted horses so that these parameters were evenly distributed between the two contrasted groups for each behavioural indicator (Supplementary Table S3, Supplementary Table S2).

Four months after the beginning of the study (T1), venous blood (10 ml) was collected once between 09:00 and 12:00 from the horses using EDTA tubes (BD Vacutainer®). In order to stabilize intracellular RNA, 0.8 ml of venous blood was transferred to a tube containing 0.8 ml of Lysis Buffer DL (Macherey-Nagel, Düren, Germany). After homogenization, the samples were maintained at −20 °C until RNA extraction. Total RNA was extracted using the NucleoSpin® RNA Blood kit (Macherey-Nagel, Düren, Germany). The manufacturer's protocol was modified to obtain sufficient ARN quantities for the microarray gene expression analyses: extraction was carried out from 1.6 ml of the mixed blood and Lysis Buffer DL. Twenty µl of proteinase K was then added to complete blood lysis. To adjust RNA binding conditions, 0.8 ml of 70% ethanol was added. The following steps were not modified, except the elution that was carried out with 40 µl of RNase-free water. Total RNA quality was assessed using RNA Pico chips on a Bioanalyzer 2100 (Agilent, Boeblingen, Germany), and its concentration was measured on a NanoDrop One Spectrophotometer (ThermoScientific, Illkirch, France).

2.4. Microarray gene expression analyses

Gene expression profiles were performed at the GeT-TRiX facility (GénoToul, Génopole Toulouse Midi-Pyrénées) using Agilent Sureprint G3 Horse_60 K_2016_01_22 021322 GE microarrays (8 × 60 K, design AMADID 081421) following the manufacturer's instructions. For each

sample, Cyanine-3 (Cy3) labelled cRNA was prepared from 50 ng of total RNA using the One-Color Quick Amp Labeling kit (Agilent Technologies) according to the manufacturer's instructions, followed by Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, Massachusetts). Dye incorporation and cRNA yield were checked using Dropsense™ 96 UV/VIS droplet reader (Trinean, Belgium). A total of 600 ng of Cy3-labelled cRNA were hybridized on the microarray slides following the manufacturer's instructions. Immediately after washing, the slides were scanned on Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software. The fluorescence signal was extracted using Agilent Feature Extraction software v10.10.1.1 with default parameters.

Microarray data and experimental details are available in NCBI's Gene Expression Omnibus [39] and are accessible through GEO Series accession number GSE215200 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE215200).

2.5. Impact of blood cell proportions in gene expression profiles

We assessed whether the observed gene expression changes in the different analyses (High *vs.* Low) were related to changes in cell proportions in the blood samples using the Celltype Computational Differential Estimation CellCODE R package [40].

2.6. Blood haematological assays and faecal measurements

As described by Ref. [19], blood samples were also collected at the beginning of the first month (T0) and at the end of the ninth month (T2) of the behavioural analysis part of the study. Whole blood samples were taken in EDTA and lithium heparin tubes (BD Vacutainer ®, 10 mL) for haematological assays. Blood was stirred for 15 min at room temperature to facilitate oxygenation. The erythron was assessed from peripheral blood samples by calculating the number of circulating red blood cells (RBC), haemoglobin concentration (HB), packed cell volume (PCV), volumetric indices, such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). The leukon was assessed from data derived from the total and differential count of white blood cells (WBC) and the analysis of WBC morphology. Different WBCs were analysed, including leucocytes (lymphocytes (LYM), monocytes (MON), neutrophils (NEU), basophils (BAS), and eosinophils (EOS)). The total blood cells were counted with an MS9-5 Hematology Counter® (digital automatic hematology analyzer, Melet Schloesing Laboratories, France).

Fresh faecal samples from each animal were collected from the rectum at T0 and T2, as described in our previous studies. Approximately 10 g of faeces were collected from the center of several faecal balls, avoiding collection of faecal material that was touching the veterinarian globes. Faecal aliquots for microbiota analysis were immediately snap-frozen in liquid nitrogen and stored at −80 °C until DNA extraction, whereas faecal aliquots to measure the faecal pH were immediately sent to the laboratory. Total DNA was extracted using the EZNA Stool DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA) following the manufacturer's instructions. DNA was then quantified using a Qubit and a dsDNA HS assay kit (Thermo Fisher).

The V3–V4 hyper-variable region of the 16 S rRNA gene was amplified, as previously reported by our team [41]. The concentration of the purified amplicons was measured using a Nanodrop 8000 spectrophotometer (Thermo Fisher) and their quality was checked using DNA 7500 chips onto a Bioanalyzer 2100 (Agilent Technologies). All libraries were pooled at equimolar concentration, and the final pool had a diluted concentration of 5 nM and was used for sequencing. The pooled libraries were mixed with 15% PhiX control according to the protocol provided by Illumina (Illumina, San Diego, CA, USA) and sequenced on a single MiSeq (Illumina, USA) run using a MiSeq Reagent Kit v2 (500 cycles).

The Divisive Amplicon Denoising Algorithm (DADA) was implemented using the DADA2 plug-in for QIIME 2 (version 2019.10) to perform quality filtering and chimaera removal and to construct a feature table consisting of read abundance per amplicon sequence variant (ASV) by sample [42]. DADA2 models the amplicon sequencing error to identify unique ASV and infers sample composition more accurately than traditional Operational Taxonomic Unit (OTU) picking methods that identify representative sequences from clusters of sequences based on a % similarity cut-off [42]. The output of DADA2 was an abundance table in which each unique sequence was characterised by its abundance in each sample. Taxonomic assignments were given to ASVs by importing SILVA 16 S representative sequences and consensus taxonomy (release 132, 99% of identity) to QIIME 2 and classifying representative ASVs using the naive Bayes classifier plug-in [43]. The feature table, taxonomy, and phylogenetic tree were then exported from QIIME 2 to the R statistical environment and combined into a phyloseq object [44]. Prevalence filtering was applied to remove ASVs with less than 1% prevalence and in fewer than three individuals, decreasing the possibility of data artefacts affecting the analysis [42]. To reduce the effects of uncertainty in ASV taxonomic classification, we conducted most of our analysis at the microbial genus level.

The phyloseq (version 1.32.0) [45], vegan (version 2.5.6) [46] and microbiome packages (version 1.10.0) were used in R (version 4.0.2) for the downstream steps of analysis. The abundance of data was aggregated at genus, family, order, class and phyla levels throughout the taxonomic-agglomeration method in the phyloseq R package, which merges taxa of the same taxonomic category for a user-specific taxonomic group. The genera abundance data were scaled to proportions to correct the differences in read depth. This processing step was performed by scaling the reads for each taxon in a given sample by the total number of reads in that sample.

2.7. qPCR quantification of the bacterial, fungal, and protozoan concentration

The concentrations of bacteria, anaerobic fungi, and protozoa in faecal samples were quantified by qPCR using a QuantStudio 12 K Flex platform (Thermo Fisher Scientific, Waltham, USA). Primers for real-time amplification of bacteria (FOR: 5′-CAGCMGCCGCGGTAANWC-3'; REV: 5′-CCGTCAATTCMTTT-RAGTTT-3′), anaerobic fungi (FOR: 5′-TCCTACCCTTTGTGAATTTG-3'; REV: 5′-CTGCGTTCTTCATCGTTGCG-3′) and protozoa (FOR: 5′-GCTTTCGWTGGTAGTG-TATT-3'; REV: 5′-CTTGCCCTCYAATCGTWCT-3′), are described in Refs. [47,48]. Further details are reported by Ref. [41].

2.8. Statistical analyses

Microarray data were analysed using R (R version 4.1.3, R Core Team, 2018) and Bioconductor packages (http://www.bioconductor.org, v.3.15) [49] as described in GEO accession GSE215200. Raw data (median signal intensity) were filtered, $\log_2$ transformed, corrected for batch effects (microarray washing bath serials), and quantile normalized [50] using the limma package (v.3.50.3) [51].

The differential analysis was performed using the limma R package. A linear model was fitted for each gene using the limma lmFit() function [51], considering microarray slide positions as a blocking factor. Pair-wise comparisons between contrasted horses for each behavioural indicator (High *vs* Low, Supplementary Fig. S1) were applied using specific contrasts. As previous analyses showed that some factors specifically influence each behavioural indicator [4], contrasted horses from experimental groups were balanced for these factors as shown in Supplementary Table S3, Supplementary Table S2. A correction for multiple testing was applied using the Benjamini-Hochberg procedure (BH) [52], to control the False Discovery Rate (FDR). Probes with FDR $\leq$0.05 were considered to be differentially expressed between conditions.

For the annotation of the Agilent chip probes, we used the annotation provided by Agilent and for the non-annotated probes, the annotation

was performed using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/). These BLASTs were performed with the following parameters: Organism = *Equus caballus*; minimum cover = 95%, minimum identity = 95%. The relationships between gene expression and welfare indicators were analysed using the R package mixOmics v.6.18.1 [53]. Principal component analysis (PCA), sparse Partial Least Square (sPLS), and sparse Partial Least Square Discriminant Analysis (sPLSDA) were performed to explore the data set structure, characterise the correlation between gene and behavioural indicators expressions and identify the genes that contributed the most to explaining differences between contrasted horses for each behavioural indicator (High *vs* Low, Supplementary Fig. S1). The functions of the genes selected through sPLS and sPLSDA were annotated using GeneCards (http://www.genecards.org/), and each list of genes was analysed using Ingenuity Pathway Analysis (IPA, QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

A gene set enrichment analysis using the R package GAGE [54] was also performed to identify if genes belonging to specific GO terms or KEGG pathways were over-represented in the gene data set. Significance was set at an FDR ≤0.05.

2.9. Biochemical, microbiota and behavioural data integration was carried out using several approaches and different combinations of data sets

Before the integration, we applied an additional pre-processing step for the biochemical assay data, faecal microbiota and behavioural expression data. In particular, to eliminate intra-individual variability and focus on the differential signals between T1 and T0, we considered Δ values (T1–T0) for each of these data sets, as described by Ref. [41].

The integration of data was then performed using two different methods and working with all data sets available, namely: (1) whole blood transcriptome; (2) Δ values of faecal 16 S rRNA gene sequencing data; (3) Δ values of the biochemical assay metabolites; (4) Δ values of the behavioural expression; and (5) Δ values of the concentration of faecal microorganisms.

As a first integration approach, a global non-metric multidimensional scaling (NMDS) ordination was used to extract and summarise the variation in blood genes (the "response variable") using the "metaMDS" function in the vegan R package [55]. The stress value was calculated to determine the dimensions for each NMDS. Stress value measures how much the distances in the reduced ordination space depart from the distances in the original *p*-dimensional space. High-stress values indicate a greater possibility that the structuring of observations in the ordination space is entirely unrelated to the actual full-dimensional area.

The other data sets (the "explanatory variables") were then fitted to the ordination plots using the "envfit" function in the vegan R package [55] with 10,000 permutations. The "envfit" function performs multivariate analysis of variance (MANOVA) and linear correlations for categorical and continuous variables. The effect size and significance of each covariate were determined by comparing the difference in the centroids of each group relative to the total variation, and all of the *p*-values derived from the "envfit" function were Benjamini-Hochberg adjusted. The obtained r^2 gives the proportion of variability (that is, the main dimensions of the ordination) that can be attributed to the explanatory variables.

The *N*-integration algorithm DIABLO of the mixOmics R package was used as a second integration approach. It is to be noted that, in the case of the N-integration algorithm DIABLO, the variables of all the data sets were also centred and scaled to unit variance before integration. In this case, the relationships among all data sets were studied by adding a categorical variable, i.e., the stereotypical behaviour of horses. For this analysis, 11 horses with missing values for one or more covariables were excluded. We also excluded one horse which was considered as an outlier. Thus, the analysis was performed on 33 horses. Horses with no stereotypical behaviours during the experiment ($n = 20$) were compared to horses that had expressed stereotypical behaviour at least once across the investigation ($n = 13$). DIABLO seeks to estimate latent components by modelling and maximising the correlation between pairs of pre-specified datasets to unravel similar functional relationships between them [56]. A complete weighted design was considered, and the "block.splsda" function was used to predict the number of latent components and discriminants. The model was first fine-tuned using leave-one-out cross-validation by splitting the data into training and testing. Then, classification error rates were calculated using balanced error rates (BERs) between the predicted latent variables with the centroid of the class labels (i.e., stereotypies vs non-stereotypies) using the "max. dist" function. BERs account for differences in the number of samples between different categories. Only interactions with $r \geq |0.70|$ were visualised using CIRCOS.

3. Results

3.1. Correlation between behaviour and transcriptomic data

In the first analysis, we categorized the horses according to a global welfare score combining the scores obtained for each of the four behavioural indicators, i.e., stereotypies, aggressiveness towards humans, unresponsiveness to the environment, and alert postures. We reasoned that the horses cumulating the highest scores in the various behavioural troubles would display the highest welfare impairment. We found no differentially expressed probes using a false discovery rate of 5% or a threshold non-adjusted p-value <0.005 when comparing the 2 groups of the contrasted horses. To gather information about the global structure of the behavioural indicators, a Principal Component Analysis (PCA) was carried out on the expressed behaviours for the 187 horses. We observed that the behavioural indicators were independent and opposed along the two first components, with the first component accounting for 27% of the total variation (Supplementary Fig. S2). This result suggests that combining the scores of the four behavioural indicators may mask genes' influence on the expression of a particular behaviour because genes correlated to a given behavioural indicator may show an opposed tendency with another behavioural indicator.

Thus, we performed transcriptome differential analyses considering the scores of each behavioural indicator separately. For aggressiveness towards humans, two contrasted groups of eight horses with high (7.3 ± 4.0% of scans) and low scores (0.0 ± 0.0%) were selected. In contrast, nine and eight horses per group were chosen for alert posture (high 7.7 ± 2.3%; low 0.0 ± 0.0%) and unresponsiveness to the environment (high 9.3 ± 1.0%; low 0.2 ± 0.4%), respectively. As there were only three horses with a high level of stereotypies, balanced contrasted groups were not available. Considering presence or absence of stereotypies did not provide significant results neither thus we no longer considered this behavioural indicator in this part of the study. The behavioural, physiological and housing parameters of the selected horses are summarised in Supplementary Table S3, Supplementary Table S2. The distribution of these horses for each of the three behavioural indicators is presented in Supplementary Fig. S1. We found no significant changes in immune cell subtypes' proportion in the different analyses using CellCODE (Supplementary Table S4, Supplementary Table S3). At an FDR ≤5%, no differentially expressed probes were detected for aggressiveness towards humans or unresponsiveness to the environment and only one probe for alert posture. Relaxing the threshold to a non-adjusted *p*-value of 0.005 provided 0, 3, and 24 differentially expressed probes for each of the three above-mentioned indicators. Because this number of differentially expressed probes is too low for the downstream bioinformatic analyses, we then used sparse least squares (sPLS) regression analyses to identify the most correlated (correlation value ≥ 0.5) genes with each of the three behavioural indicators using the three groups of horses. As shown in Fig. 1, genes' highest levels of correlation were found with aggressiveness towards humans, whatever the horses' selection. Arising from these results, we

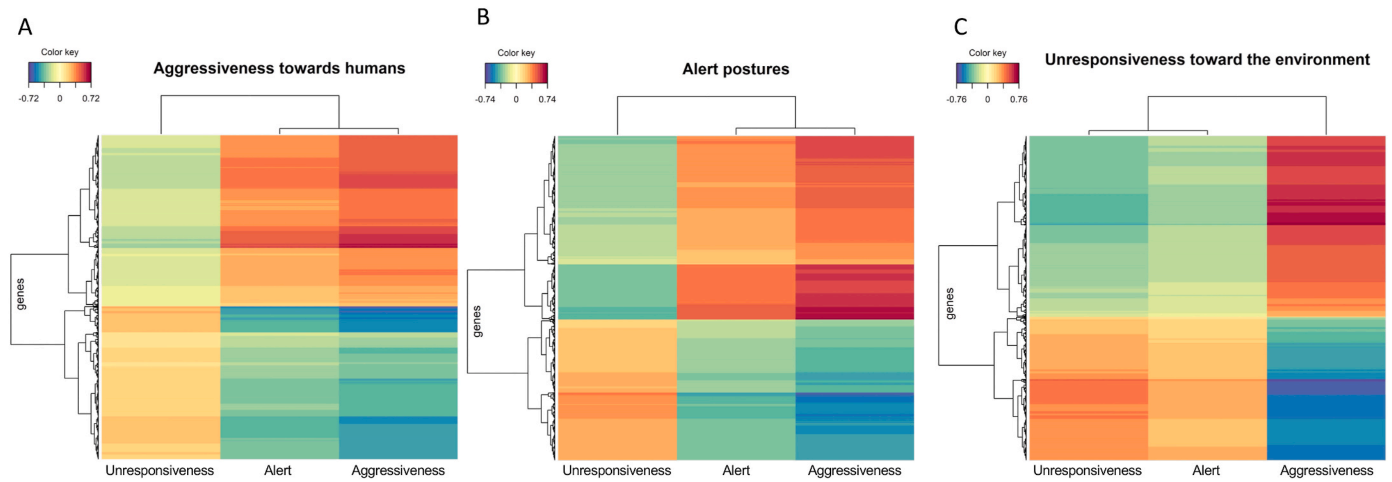

Fig. 1. Heatmaps from sPLS analyses performed on contrasted horses for the behavioural indicators (Aggressiveness towards humans (A); Alert postures (B), Unresponsiveness to the environment (C)). The plots display the correlation levels between gene expression and the behavioural indicators variables. Genes are clustered according to the direction and the intensity of the correlation with behavioural indicators. The most positive and negative correlations are represented in dark red and dark blue respectively as it is indicated in the color key legend.

focused our downstream analysis on the “aggressiveness towards humans” trait.

Within the sPLS analysis for “aggressiveness towards humans”, we detected 356 probes correlated with this behaviour which correspond to 205 unique genes. Within those genes, 99 were negatively and 106 positively correlated (correlation value $\geq$ 0.5). We then used Ingenuity Pathway Analysis (IPA) software to understand these genes’ functional implications better and reveal the molecular pathways and up-regulators underpinning this gene set. Supplementary Fig. S3 shows that the over-represented biological process terms were strongly associated with inflammatory response (e.g., cytokines IL-1A, IL1B, IL-5 expression, T cell responses, adhesion of immune cells) as well as cell differentiation pathways (differentiation of embryonic tissue, tumour cell lines, bone cells, etc.). Using the upstream regulator analysis of the IPA software, we detected 64 upstream regulators with a p-value less than 0.001 (Supplementary Table S5, Supplementary Table S4). These regulators were related to the onset of inflammation, apoptosis, and cell differentiation/growth. The list of estimated up-regulators is summarised in Fig. 2A through a CIRCOS plot. IPA analysis also provided a network of the genes positively associated with aggressiveness towards humans. Fig. 2B shows that these regulators act in a feed-forward and feedback manner to control the spatiotemporal expression patterns of

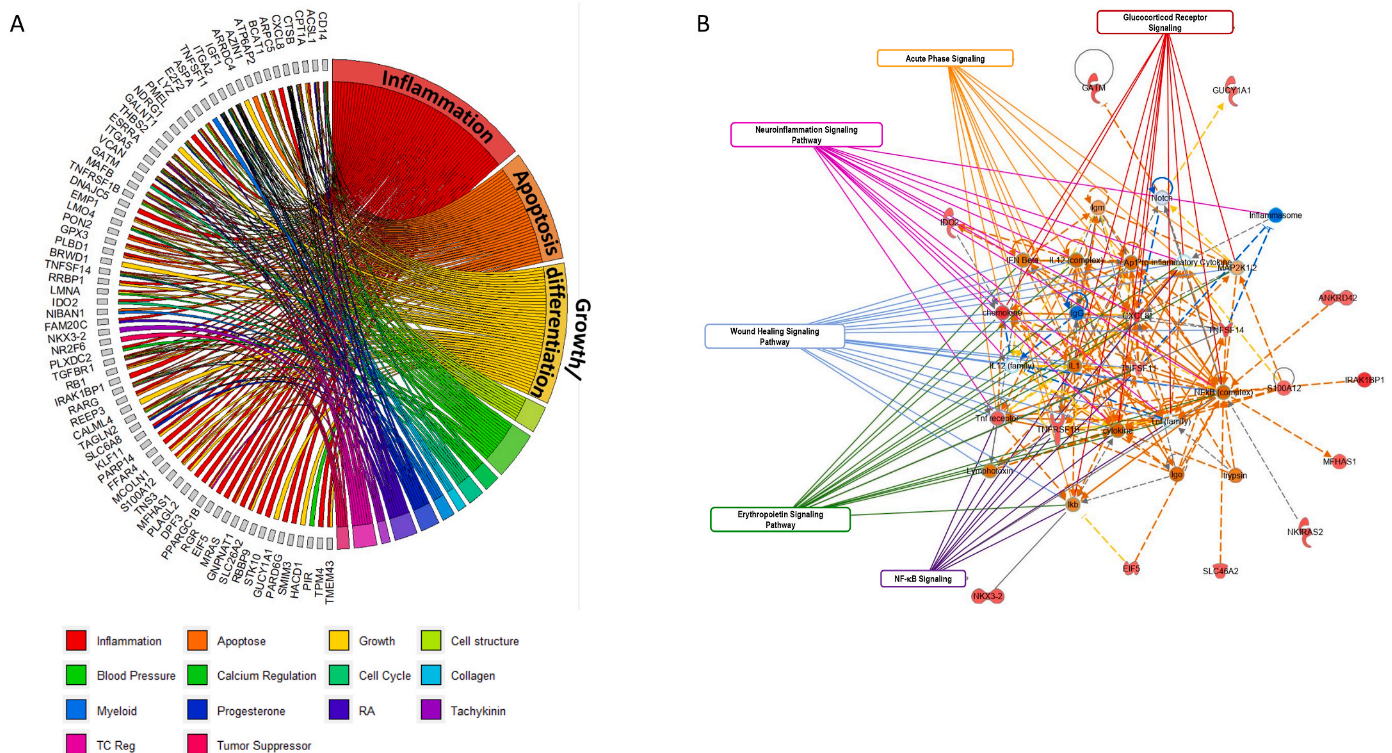

Fig. 2. Gene expression positively correlated to aggressiveness towards humans A) CIRCOS plot of main upregulators and their target genes positively correlated to aggressiveness towards humans. B) Network of genes positively correlated to aggressiveness towards humans ($r > 0.5$) generated through the use of QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA).

pathways related to inflammation and immune responses (e.g., NFKB signalling, acute phase response signalling, and wound healing signalling). Next, we performed a sPLSDA (sparse least-square discriminating analysis) in which the horses with high or low aggressiveness scores were considered a discriminatory category (Fig. 3A) contrary to the sPLS analysis, where the quantitative scores of aggressiveness were considered. We detected 158 probes correlated with this behaviour, which correspond to 110 unique genes. Within those genes, 51 were negatively correlated and 59 positively correlated. Similar to sPLS, the most relevant cellular activities controlled by the 51 positively correlated genes were inflammatory-related pathways, e.g., NFKB, wound healing and neuroinflammation and glucocorticoid receptor signaling (Fig. 3B). However, only six genes were shared between the sPLS and sPLSDA analyses. Finally, we performed a gene set enrichment analysis (GSEA) using KEGG (Kyoto Encyclopedia of Genes and Genomes), which again confirmed the importance of the inflammatory pathways and immune responses in the expression of aggressiveness towards humans (Table 1).

3.2. *Integration of blood transcriptome and biochemical parameters, behavioural expression, faecal microbiota and microorganism concentrations*

Given that gut microbiota is considered a central organ because of its direct and indirect roles in horse physiology, including improved metabolic health and welfare [18,19], we tethered the whole blood transcriptome profiling and behaviour data to the gut ecosystem. By jointly characterising the whole blood transcriptome, biochemical parameters, behaviour and faecal microbiota of 45 horses, we aimed to improve our understanding of the holobiont under behaviour impairment.

To this aim, we applied two independent statistical methods using the whole blood transcriptome as the response variable and the other data sets, namely biochemical assay profiles, the faecal microbiota composition, the concentrations of bacteria, anaerobic fungi, and protozoa and the frequencies of the behavioural indicators as exploratory variables (except for stereotypies). We first used global NMDS ordinations to visualise the structure of blood transcriptome (ordination stress = 21%, $k = 2$, non-metric fit $r^2 = 0.\ 0.954$), and we then fitted all sets of

Table 1
Enrichment analysis using KEGG pathways performed on contrasted horses for aggressiveness towards humans.

Global behavioural score		
KEGG pathways	Class	FDR q-value
hsa04640 Hematopoietic cell lineage	Organismal Systems; Immune system	1,80E-08
hsa04060 Cytokine-cytokine receptor interaction	Environmental Information Processing; Signaling molecules and interaction	1,43E-07
hsa04610 Complement and coagulation cascades	Organismal Systems; Immune system	1,45E-05
hsa04061 Viral protein interaction with cytokine and cytokine receptor	Environmental Information Processing; Signaling molecules and interaction	2,10E-05
hsa03320 PPAR signaling pathway	Organismal Systems; Endocrine system	1,09E-03
hsa04512 ECM-receptor interaction	Environmental Information Processing; Signaling molecules and interaction	2,08E-03
hsa04657 IL-17 signaling pathway	Organismal Systems; Immune system	3,04E-03
hsa04145 Phagosome	Cellular Processes; Transport and catabolism	9,55E-03
hsa04080 Neuroactive ligand-receptor interaction	Environmental Information Processing; Signaling molecules and interaction	1,25E-02
hsa04810 Regulation of actin cytoskeleton	Cellular Processes; Cell motility	1,84E-02
hsa04510 Focal adhesion	Cellular Processes; Cellular community - eukaryotes	4,58E-02
hsa04668 TNF signaling pathway	Environmental Information Processing; Signal transduction	4,58E-02
hsa04672 Intestinal immune network for IgA production	Organismal Systems; Immune system	6,21E-02
hsa04620 Toll-like receptor signaling pathway	Organismal Systems; Immune system	9,45E-02

explanatory variables to the ordination to find the most influential variables (Supplementary Table S6, Supplementary Table S5). Bacteria such as *Prevotella, Eggerthella, Succinivibrio, Paraprevotella, Ruminobacer,*

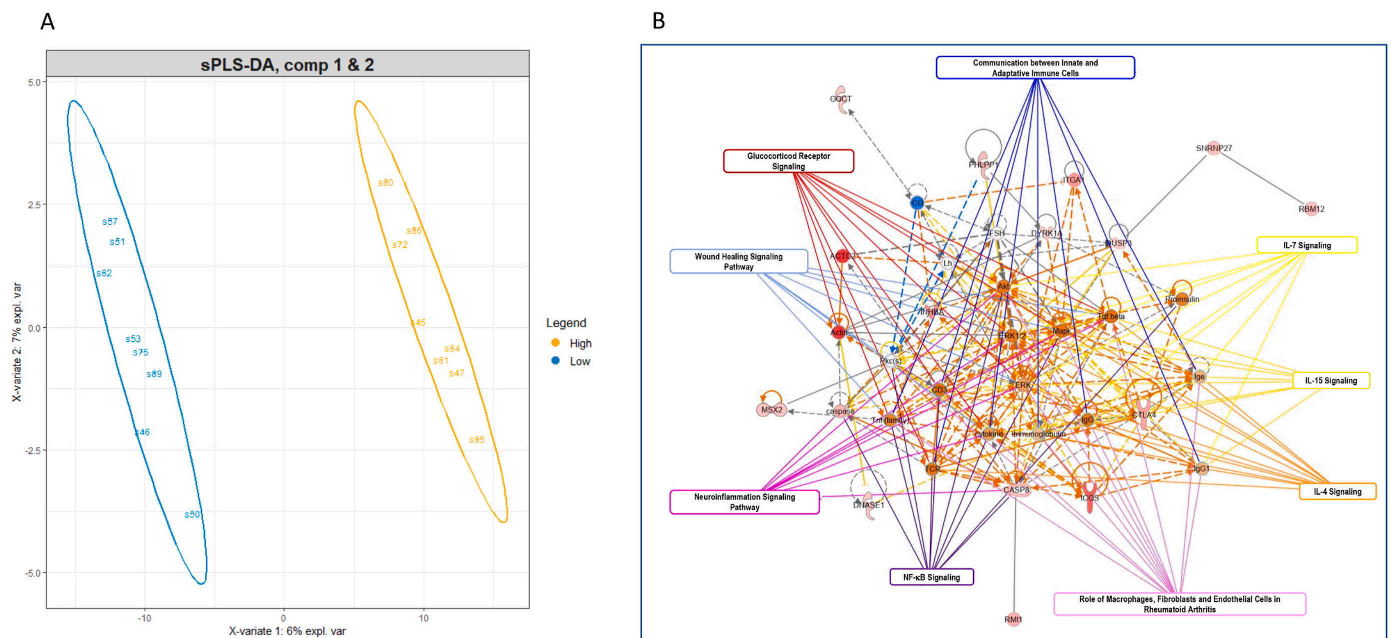

Fig. 3. sPLSDA analysis of aggressiveness towards humans A) Sample plots from sPLSDA performed on the gene expression data, including 95% confidence ellipses. Samples are projected into the space spanned by the first two components and coloured by their aggressiveness subtypes. B) Network of genes positively correlated to aggressiveness towards humans ($r > 0.5$) generated through the use of QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA).

Rikenella, Treponema, Rummeliibacillus and Clostridia members *(Flavonifractor, Clostridium III, Anaerostipes, Lachnospira)* showed the strongest correlation to all ordinations, together with blood biochemical parameters (e.g., haematocrit: haemoglobin ratio, and % of monocytes) and the presence or absence of stereotypies (adjusted $p < 0.05$; Fig. 4).

Because the presence/absence of stereotypies was a primary driver of the overall structural variation of the blood transcriptome (*envfit*, $R^2 = 0.193$, adjusted $p = 0.040$; (Supplementary Table S6, Table S5), we examined further the relationships among all the above data sets by adding the stereotypies as a categorical variable. We used the DIABLO framework from mixOmics [56]. While the blood transcriptome showed high levels of covariation with the faecal microbiota ($r^2 = 0.84$, Supplementary Fig. S4), it was impossible to identify a tight relationship with the other data sets. A more fine-grained view of this biological system was then obtained by focusing on pairwise correlations between variables. The first component of the DIABLO analysis highlighted a significant link between a subset of 50 genes and ten gut bacterial taxa (e.g., the genus *Kurthia, Lysinibacillus, Prevotella, Rummeliibacillus, Solibacillus, Paraprevotella, Enterococcus, Ruminococcus, Saccharofermentans* and the family *Lachnospiraceae*; Supplementary Fig. S5). Overall, this method validated the associations already detected with the NMDS approach. Moreover, five more *Bacilli* taxa found with DIABLO appeared to be functionally related to other previously identified microorganisms, thus providing indirect support to those findings. This was the case for *Kurthia, Lysinibacillus, Rummeliibacillus, Solibacillus,* and *Enterococcus* (Fig. 5).

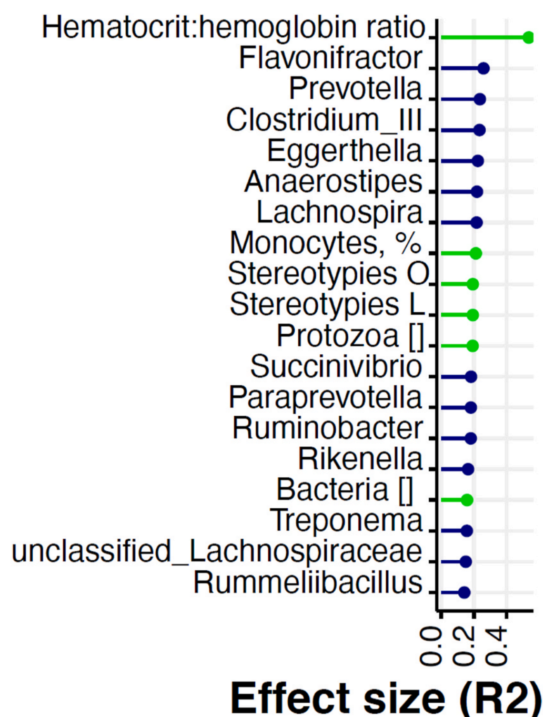

Fig. 4. Associations between genes, microbiota, behaviour, and circulating blood parameters. Effect sizes of variables over multidimensional scaling (NMDS) ordination plot. Covariates are coloured according to the dataset type: bacteria in dark blue and the other variables in green. Horizontal bars show the amount of variance (r^2) explained by each covariate in the model as determined by the 'envfit' function. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Aggressiveness towards humans is associated with transcriptional pathways of inflammation, apoptosis, and growth/differentiation

The sPLS analyses revealed that aggressiveness towards humans is strongly associated with the expression of a set of genes. In contrast, the correlations between genes, high frequencies of alert postures and unresponsiveness towards the environment were weak. Stereotypies could not be analysed appropriately by differential analyses because too few horses displayed high levels of this behaviour. Using presence or absence of stereotypies as contrasted groups did not lead to significant results neither. Interestingly, we found in a previous analysis that aggressiveness towards humans showed, compared to unresponsiveness to the environment or alert postures, a higher repeatability and convergent validity using three different methods of assessment [25]. More labile behaviours, such as unresponsiveness to the environment or alert postures, may explain why correlations with whole blood transcriptome are difficult to observe.

The bioinformatic investigations that followed the sPLS analysis revealed the importance of inflammation, apoptosis, growth, and cell differentiation. Inflammatory pathways were again highlighted after a sPLSDA analysis on aggressiveness traits. Aggressiveness or reactive aggression, defined as an impulsive response to provocations, frustrations or threats from the environment, has been studied in various species. The activity of Monoamine oxidase A (MAOA), which degrades amine neurotransmitters, such as dopamine, norepinephrine, and serotonin, via oxidative deamination, was found to correlate to aggressive behaviour in mice, including aggressiveness induced by social isolation [57] as well as in humans [58] and cattle [59]. In this study, the MAOA gene was expressed in blood, but no correlation was found with aggressiveness towards humans.

Inflammation and immune functions were the most important pathways unveiled by the sPLS or sPLSDA analyses. Other transcriptomic studies conducted in mouse models of aggressiveness [60] or in zebrafish [61] but on brain tissues, led to the same conclusions. In mice prefrontal cortex of aggressive versus non-aggressive mice, the transcriptomic analysis uncovered NFKB and MAPK pathways which are known to be both involved in inflammation [60]. In brains of aggressive vs non-aggressive zebrafishes, RNAseq analysis also unveiled an important role of the inflammatory pathway such as MAPK, involving the expression of several pro-inflammatory cytokines, chemokine receptors and other genes related to immune system activation [61]. Changes in pro-inflammatory cytokine levels have been linked to aggressiveness in animals and humans [62]. Along the same lines, a recent study found that the neutrophil to lymphocyte ratio was increased in horses that were relocated to individual stabling after six weeks housed in one social group on pasture [63]. The hypothesis is that it is beneficial for the organism to up-regulate the immune functions during and after stressful and/or aggressive encounters where physical injuries are likely to occur. The up-regulation of the "wound healing pathway" found in the sPLS and sPLSDA analyses favour this hypothesis. The glucocorticoid signalling pathway was also found to be up-regulated in these analyses. Although a causal link between aberrant hypothalamic-pituitary-adrenal (HPA) axis and aggressiveness has not been proved so far, developmentally low or high HPA axis reactivity is typically found to be aligned with the emergence of aggressive phenotypes [64]. Exposure to stressful experiences during different stages of development is known to have long-term consequences on HPA axis function and behaviour. For instance, sudden and early weaning is associated with increased reactivity to humans and long-term elevated cortisol levels compared to foals progressively separated from their mother [22]. Such weaning practice, in addition to the housing conditions in individual stalls, may have favour aggressiveness towards humans in part of the horses of this study.

Finally, pathways related to apoptosis as well as cell growth and

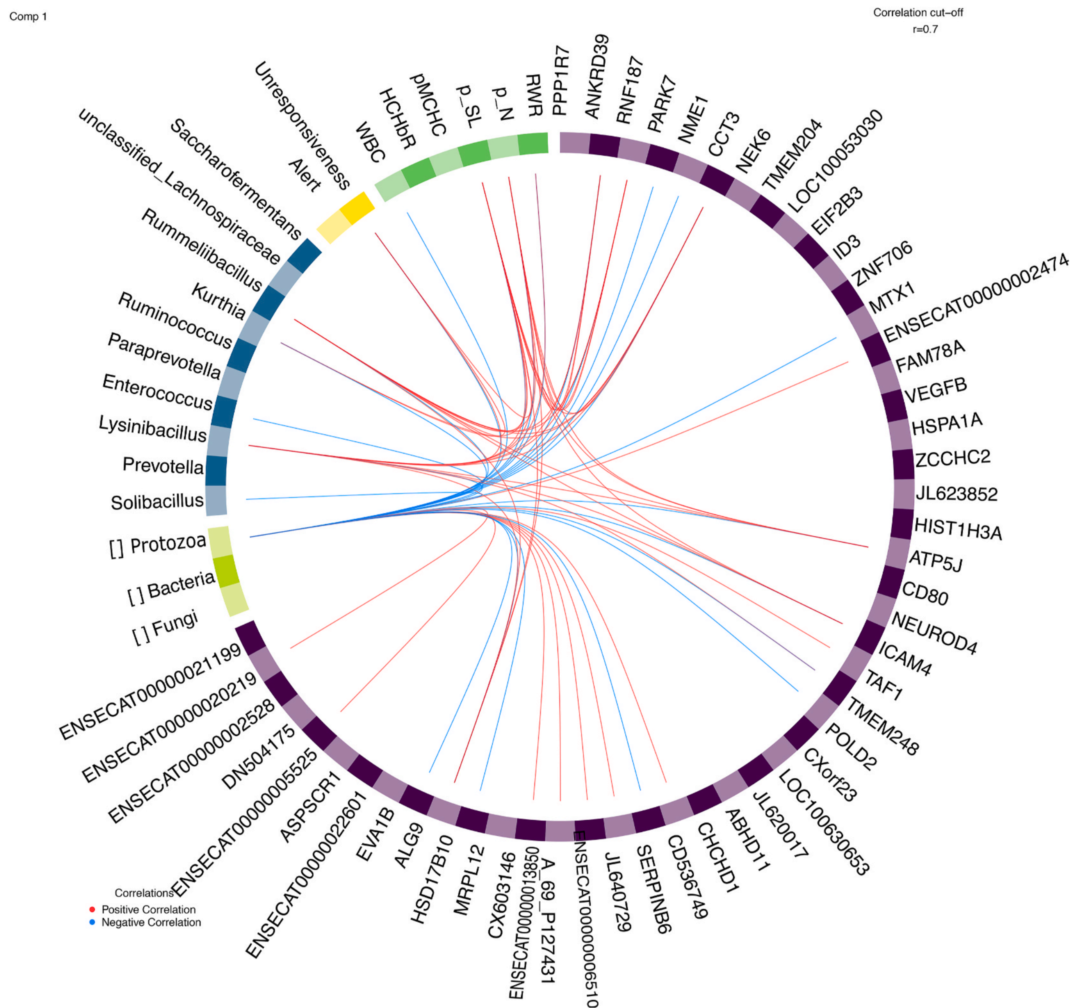

Fig. 5. A variable plot of genes, fecal microorganisms' quantification, bacteria, behaviours, and hematological parameters that predict the presence or absence of stereotypies in horses. Circos plot demonstrates the bio-signature from multiple datasets over the two components. Each dataset is given a different color. Transcriptome data is represented in violet, fecal microorganisms' quantification in light green, bacteria in blue, behaviours in yellow and hematological parameters in light green. The red and blue lines indicate positive and negative correlations between the two variables ($r \geq |0.70|$), providing information on which an explanatory relationship likely links variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

differentiation, were uncovered by the sPLS (but not sPLSDA) analysis. In a study comparing horses kept alone in individual boxes with horses submitted to a socially and physically enriched environment for 12 weeks, apoptosis pathways were associated with the first condition [23], in agreement with the data from this study. The gene expression correlation with cell growth and differentiation, especially bone cells (osteoblasts), is more difficult to interpret. However, in some inflammatory conditions such as ankylosing spondylitis abnormal bone formation occur through the activation of Bone Morphogenetic Protein (BMP) transcription factors [65] that was found positively correlated with aggressiveness towards human in this study.

Importantly for the health of horses, aggressiveness towards humans was previously found to be correlated with inflammatory conditions such as chronic back pain [32], various injuries [31] and lameness [66].

4.2. Integration of faecal microbiota composition and gene expression is associated to the expression of stereotypies

Because faecal microbiota and circulating biochemical parameters had been measured on the same 45 horse population, we combined these datasets with the transcriptomic analysis and the behavioural indicators to characterise the holobiont. These analyses did not detect associations between whole blood transcriptome, gut microbiota composition and aggressiveness. This was not unexpected because using the full cohort of individuals ($n = 187$ [19]) the "Aggressiveness towards humans" did not relate to overall gut microbiota composition. Still, two lactate-producing bacteria (*Streptococcus* and *Butyrivibrio)* were correlated to aggressiveness [19]. This correlation was not found here, probably due to the lower number of horses analysed. By contrast, presence or absence of stereotypies were correlated to gene expression when combined to microbiota composition in the subpopulation analysed here as expected from the whole horse population study, which detected stereotypies as the behavioural indicator that correlated the most to microbiota composition [19]. However, *Ruminobacter* was the only bacteria genus in common between the two analyses. As for the gene set correlated to stereotypies, we did not detect a functional network or apparent pathways that would describe the whole gene set. Among the gene positively correlated to stereotypies, CD80 is involved in the production of cytokines, while ZCCHC2, CXorf23, and TAF1 have a role in the positive regulation of gene transcription. Interestingly, a SNP within the ZCCH2 gene was found to be linked to depressive symptoms in human [67] and TAF1 was shown to play a role in the apoptosis of endothelial cells under high glucose conditions [68]. Further work on these genes is required to explain their relationship with stereotypies in horses. Finally, the

percentage of monocytes was also correlated to stereotypies, suggesting a higher inflammatory status in horses with stereotypies, fitting with the expression of CD80 and maybe with TAF1.

5. Conclusion and practical implications

This study shows that aggressive and stereotypic horses are not only “nasty horses” or “badly educated”.

We have highlighted physiological correlates in blood gene expression and in their faecal microbiota composition that support the fact that these horses are indeed experiencing physical suffering and compromised welfare. These results add to the existing body of knowledge on these two indicators. On the contrary, further studies are needed on the higher expressions of "unresponsiveness to the environment" and "alert postures” and their relationships with transcriptomic data. Indeed, this work has several limitations that may explain the lack of correlation with the transcriptome for these latter traits. First, these two behavioural alterations, measured twice, showed weak correlations between the two periods of measurements [25]; second, the scan sampling method used, in particular for short-term behaviour such as alert posture may not be the most appropriate; third, the horse sample for each behavioural indicator is small for sPLS regressions because they were first selected for differential gene expression analyses, which may also explain the discrepancy with the data made on 45 horses when transcriptomic was combined to biochemical and microbiota data; fourth, the annotation of the horse genome is not complete which inevitably reduce the chance to obtain significant results. However, the latter is going to improve with the recent efforts made in annotating the horse genome through the Functional Annotation of the Animal Genomes (FAANG) project [69].

Ethics approval

Our study protocol was assessed and approved by the local animal care and use committee (CEEA Val de Loire; reference: 2019012211274697.V4-18939). The protocol followed EEC regulation (no 2010/63/UE) governing the care and use of laboratory animals, which has been effective in France since the 1st of January 2013. Participants provided their verbal informed consent for animal blood and fecal sampling at each time point as well as for the related survey questions. The blood and fecal samples were collected by veterinarians adhering to the regulations and guidelines on animal husbandry and welfare.

Data and model availability statement

Microarray data and experimental details are available in NCBI’s Gene Expression Omnibus [39] and are accessible through GEO Series accession number GSE215200 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE215200).

This Targeted Locus Study project has been deposited at DDBJ/EMBL/GenBank under the BioProject PRJNA543287. The corresponding BioSamples accession numbers were SAMN11666402 to SAMN11666784.

Author contributions

MPM, LL, and NM design the experiments. AF and AR did the experiments. MPM, AF, AR, NM, and LL wrote the manuscript.

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Declaration of competing interest

The authors have no interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cpnec.2023.100201.

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