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Original Research

Tissue-Specific Variation in Aquaporins and Cytokines Transcriptional Profiles in Piglets being LPS Challenged

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Abstract

Background: Lipopolysaccharide (LPS), an effective stimulator of the immune system, has been widely applied in an experimental pig model for human sepsis. Aquaporins (AQPs), a family of small integral membrane proteins responsible for facilitating water fluxes through the cell membrane, offer potential promising drug targets for sepsis treatment due to their role in water balance and inflammation. **Methods**: In order to investigate the potential effect of a dietary amino acid mixture supplementation on LPS-challenged weaned piglets, a total of 30, 28-day-old, males were randomly allocated to 1 of 3 dietary treatments for a 5-week period, with 10 animals in each: diet 1 was a control (CTL) treatment; diet 2 was LPS treatment, where the piglets were intraperitoneally administered LPS (at 25 µg/kg body weight); diet 3 was LPS + cocktail treatment, where the piglets were intraperitoneally administered LPS and fed a diet supplemented with a mixture of arginine, branched-chain amino acids (BCAA, leucine, valine, and isoleucine), and cystine. Key organs that control sepsis were collected and processed by real time quantitative PCR (RT-qPCR) for the AQPs and cytokines transcriptional profiles. **Results**: Minor variations were detected for AQPs and inflammatory markers mRNA levels, upon the dependence of LPS or the amino acid cocktail suggesting the piglets' immune recovery. Using a discriminant analysis tool, we report for the first time, a tissue-specific variation in AQPs and cytokines transcriptional profiles that clearly distinguish the small intestine and the kidney from the liver and the spleen. **Conclusions**: This study provides a novel insight into the gene expression signature of AQPs and cytokines in the functional physiology of each organ in piglets.

Keywords: lipopolysaccharide; sepsis; aquaporins; inflammation; amino acids; piglets

1. Introduction

Sepsis is a common cause of death in intensive care units all over the world and it is associated with antibiotic resistance and the lack of an appropriate antimicrobial therapy [1]. Lipopolysaccharide (LPS)-induced chronic inflammation has been successfully used to mimic sepsis in studies conducted *in vitro* and *in vivo* [2,3]. Due to the complexity of this immunological syndrome implying higher mortality rates, new therapeutic strategies, and predictive biomarkers are of the utmost importance [4–6]. In this regard, aquaporins (AQPs), which are passive transporters of water, are potentially promising drug targets since they play a relevant role in inflammation and particularly in sepsis, where disrupted water balance is observed [7,8]. In fact, each of the 13 AQPs detected in humans has particular features that make them crucial players in several physiological roles, including immunity and inflammation, being involved in signaling cascades for volume regulation, regulating the subcellular localization of other proteins, and cell adhesion [9,10]. Beyond water homeostasis, evidence showed that AQPs can also facilitate the transport of small non-charged solutes (such as glycerol, urea, and hydrogen peroxide), gases (CO_2), and cations [9,11]. Elucidating the full range of functional roles for AQPs beyond the passive conduction of water will improve our understanding of mammalian physiology in health and disease. The functional variety of AQPs makes them an exciting drug target and could provide routes to a range of novel therapies.

LPS initially acts on the local immune system, leading to water influx (diarrhea) as a direct response of AQP1 to the antigen in the small and large intestines [12]. Moreover, AQP3 is diminished in the intestinal epithelium of LPS-treated mice [13]. The liver is also a preferential target for sepsis-induced injury. AQP8 gene expression is reduced in hepatocytes [14] causing reduced bile formation and aggra-

vated cholestasis, while AQP9 remained unaffected [15]. Another common complication in sepsis is acute kidney injury, which often leads to increased mortality owing to water imbalance. Downregulation of AQP2 was shown in animal models using LPS after a short-time exposure [16], whereas, after a long-time exposure, AQP2 was upregulated in the kidney [17]. AQP9 immunolabeling confirmed granulocytes or monocytes as being responsible for this AQP isoform expression in the spleens of rats [18]. Despite little being known regarding the modulation of AQPs, the activation of the proinflammatory c-Jun N-terminal kinase (JNK) and/or nuclear factor-kappa B (NFκB) pathways have been described as being able to modulate the gene expression of AQPs in adipocytes. Moreover, these same pathways impact the transcription of proinflammatory markers, such as tumor necrosis factor-alpha (TNFα), and interleukins (IL)-1β and IL-6 [19]. LPS activates cells mainly via Toll-like receptor 4 (TLR4), which in turn triggers the mitogen-activated protein kinase (MAPK)/JNK pathway [20]. Previously, the MAPK pathway was shown to modulate AQPs expression. The use of MAPK pathway inhibitors demonstrated that p38 and JNK induce AQP4 protein levels in cultured human primary cortical astrocytes [21]. Moreover, the expression and localization of AQP3 and AQP9 were shown to be regulated by the p38 MAPK pathway [22].

Uncovering AQPs as important druggable targets highlights the utmost need to develop AQP-targeted medicines with the potential to be successfully used in human therapy. Therefore, unveiling the mechanistic pathways in which AQPs are enrolled provides information on alternative routes for targeting AQPs that go beyond the traditional pore-blocking approaches [23–25].

In addition to their constructive role in protein synthesis, amino acids are key functional and signaling molecules involved in the regulation of oxidative stress, immunity, and intestinal barrier function [26], among other processes. Dietary arginine supplementation in piglets facing LPS challenge increases villus height in the jejunum and ileum portions of the small intestine [27]. Furthermore, it impacts oxidative stress status by increasing the ferric-reducing ability in the plasma and decreasing the oxidized form of glutathione [28]. Under the same experimental model of challenge, cysteine supplementation (cysteine is oxidized to cystine) has been associated with an increase in transepithelial resistance and a reduction in the production of proinflammatory cytokines [29]. Additionally, dietary branched-chain amino acid (BCAA) supplementation is associated with an improvement in the piglets' gut morphology, as well as with an increase in the expression of amino acid transporters [30,31]. Thus, these findings indicate that these amino acids have complementary physiological modes of action and could play a protective functional effect in LPS-challenged piglets.

In this study, we mimicked a bacterial infection in recently weaned piglets through the intraperitoneal administration of LPS, a bacterial endotoxin. Our main objectives were: (a) to characterize the transcriptional profile of AQPs and cytokines in key organs that control sepsis, such as the small intestine, liver, spleen, and kidneys, and to observe any highly similar gene expression patterns between the tissue samples; (b) to test the impact of LPS-induced inflammation on the transcriptional profiles of AQPs and cytokines in the small intestine, liver, spleen, and kidneys; (c) to validate the hypothesis that LPS-induced modulation of the gene expression of AQPs and pro- and anti-inflammatory mediators, might be reversed by dietary amino acid (arginine, BCAA (leucine, valine, and isoleucine), and cystine) supplementations [26]. To the best of our knowledge, this is the first *in vivo* study investigating the effect of dietary supplementation of these amino acids in piglets facing an inflammatory-immunological challenge. This integrative methodological approach may elucidate the tissue-specific signaling pathways for AQPs and cytokines interplay, thus, providing a novel insight into their physiological role in the settings of inflammation and unveiling new AQP-targeting molecules that may inspire novel therapies.

2. Materials and Methods

2.1 Ethics Statement

The experimental procedures of the animal trial were carefully reviewed and approved by the Animal Care Committee of Instituto Superior de Agronomia (ISA), Universidade de Lisboa, (Lisbon, Portugal) and authorized by the National Veterinary Authority (Direcção Geral de Alimentação e Veterinária (Lisbon, Portugal), according to European Union guidelines (2010/63/EU Directive). The ethical approval code is #0421/000/000/2017. JAMP and PAL hold a FELASA grade C certificate, and DC hold a FELASA grade B certificate, which enables them to conduct animal experimentation in the European Union.

2.2 Animals, Diets, and Sampling

The experimental trial was performed at the Animal Production Sector of Instituto Superior de Agronomia (ISA), Universidade de Lisboa (Lisbon, Portugal). Thirty 28-day-old newly weaned male crossbred piglets (F2 crosses of Pietrain $\times$ F1 [Landrace $\times$ Large white crosses]) with an initial body weight of 8.2 $\pm$ 1.0 kg (mean $\pm$ SD), were randomly allocated on day 0 to different experimental diets. Piglets were submitted to one of three dietary treatments, CTL (n = 10), LPS (n = 10) and LPS + Cocktail (n = 10), during five weeks. A basal diet was formulated to contain 20% crude protein level and a level of 1.35% standardized ileal digestible Lys. The levels of standardized ileal digestible methionine, threonine, tryptophan, valine, isoleucine, leucine, histidine, and phenylalanine + tyrosine relative to lysine followed the National Research Council

Table 1. Ingredients (g.kg^{-1}) composition of diets.

	Dietary treatments	
	CTL and LPS	LPS + cocktail
Wheat	200	200
Corn	382	382
Soybean meal	268	268
Sweet dry whey	70	70
Soybean oil	30	30
L-Lys	6.4	6.4
DL-Met	2.7	2.7
L-Thr	2.8	2.8
L-Trp	1.1	1.1
L-Val	3.2	3.2
$CaCO_3$	10	10
Dicalcium Phosphate	16	16
Sodium bicarbonate	2.2	2.2
NaCl	3.0	3.0
Vitamin trace mineral mix(1)	3.0	3.0
L-Arg	0.0	1.25
L-Leu	0.0	0.50
L-Val	0.0	0.25
L-Ile	0.0	0.25
L-Cys	0.0	0.75

(1) Vitamin and trace mineral supplied per kilogram of diet: Vit. A, 25.000 IU; Vit. D3, 2.000 IU; Vit. E, 20 IU; Vit. C, 200 mg; Vit. B1, 1.5 mg; Vit. B2, 5 mg; Vit. B3, 30 mg; Vit. B5, 15 mg; Vit. B6, 2.5 mg; Vit. B9, 0.5 mg; Vit. B12, 0.03 mg; Vit. K3, 1 mg; biotin, 80 mg; choline (chloride): 300 mg; I, 1 mg as potassium iodate; Mn, 50 mg as manganese (oxide); Fe, 120 mg as ferrous carbonate; Zn, 140 mg as zinc (oxide); Cu, 160 mg as copper sulfate; Se, 0.3 mg as sodium selenite; Co, 0.5 mg as cobalt carbonate [27]. Dietary treatments: CTL, the control diet; LPS, the LPS-challenged diet; LPS + cocktail, the LPS-challenged diet supplemented with arginine, BCAA (leucine, valine, and isoleucine), and cystine mixture.

[32] recommendations. For the LPS + cocktail group, the basal diet was supplemented with a 0.3% as-fed-basis of an amino acid mixture with arginine, BCAA (leucine, valine, and isoleucine), and cystine, in a weight ratio of 42:33:25, as shown in Table 1 (Ref. [27]).

The mixture composition was derived from an initial mixture, where the arginine, BCAA, and cystine contribution was equal. Then, the cystine contribution was decreased at the expense of arginine because cystine is known to display mucolytic effects above a certain dosage [33], while arginine is well tolerated at high dosages. After 7 days, all piglets, except the ones from the CTL group, which were injected with a sterile saline solution, received a mild LPS challenge administered intraperitoneally at a dosage of 25 µg/kg according to body weight [34]. LPS, extracted and purified from *Escherichia coli* O55:B5, was acquired from Sigma-Aldrich (St. Louis, MO, USA). During the experimental trial, piglets were housed in pens with three animals, fed with pelleted feed, ad libitum, and weighed individually once a week. The health status of the piglets was monitored during the experimental trial and no casualties were observed. At the end of the experiment, piglets were euthanized by electrical stunning and exsanguination, according to EU legislation. For additional details on the experimental trial, see Prates *et al.* [35]. Samples from the ileum of the small intestine (60 cm before the ileocecal valve), liver, kidneys, and spleen were collected, weighed, flash frozen, and stored at –80 °C, until analysis. Indicators of the inflammatory status were reported previously in a companion paper by Prates *et al*. [35] and were integrated into the Discussion section.

2.3 Tissue Disruption, RNA Extraction, and cDNA Synthesis

Tissues (ileum, liver, kidney, and spleen) were homogenized in NZYol Reagent (NZYtech, Lisbon, Portugal), using a TissueRuptor II (Qiagen, Hilden, Germany), and RNA extraction was carried out, according to the manufacturer's protocol. For cDNA synthesis, 1 µg of total RNA with quality ratios 260/280 and 260/230 between 1.8–2.2 (NanoDrop1 ND-2000c, ThermoFisher Scientific, Waltham, MA, USA) were reverse transcribed in a 20 µL final volume using NZY First-Strand cDNA Synthesis Kit (NZYtech, Lisbon, Portugal), according to the manufacturer's protocol, as described by da Silva *et al.* [36].

2.4 Relative Gene Expression Analysis by Real-Time Quantitative PCR

AQPs (1, 2, 3, 5, 8, 9, and 10), inflammation key players (CCL2, CXCL8, and NLRP3), as well as cytokines (TNF-α, IL-1β, IL-6, and IL-10) gene expression levels, were evaluated in ileum, liver, spleen, and kidney samples by real time quantitative PCR (RT-qPCR), as previously described by da Silva *et al*. [36]. In brief, the quantification was performed using TaqMan™ Universal Master Mix II with UNG (Life Technologies, Carlsbad, CA, USA) and *Sus scrofa* specific predesigned TaqMan™ Gene Expression Assays (primers FWD, REV and FAM-labelled probe; Life Technologies) in a QuantStudio 7 Flex (Applied Biosystems, Waltham, MA, USA) with the QuantStudio™ Real-Time PCR Software v1.2 (ThermoFisher Scientific).

The selected target genes and corresponding primers/probes are listed in Table 2. Probes were chosen in order to hybridize between exons to guarantee the detection of the corresponding transcript avoiding genomic DNA amplification. The cDNA amplification was performed as follows: 95 °C for 10 min, followed by 40 cycles of 15 seconds at 95 °C, and 1 min at 59 °C. qPCR reactions were carried out in duplicate, and data were normalized to the housekeeping gene HPRT1, the most stable gene of internal controls for normalization. Relative expression levels were calculated using a variation of the Livak and Schmittgen [37] method, reported by Fleige and Pfaffl [38].

Table 2. Gene-specific primers and probes used for RT-qPCR.

Gene symbol	Full gene name	GenBank accession no.	TaqMan gene expression assay	Product size (bp)
AQP1	*Aquaporin-1*	NM_214454.1	Ss03385017_u1	97
AQP2	*Aquaporin-2*	EU636238.1	Ss04321195_m1	80
AQP3	*Aquaporin-3*	NM_001110172.1	Ss03389620_m1	77
AQP5	*Aquaporin-5*	EU192130.1	Ss03389675_m1	105
AQP8	*Aquaporin-8*	EU220426.1	Ss03386904_u1	60
AQP9	*Aquaporin-9*	NM_001112684.1	Ss03389741_m1	65
AQP10	*Aquaporin-10*	NM_001128454.1	Ss03374224_m1	61
IL-6	*Interleukin-6*	AB194100.1	Ss03384604_u1	76
IL-1β	*Interleukin-1 beta*	AK344440.1	Ss03393804_m1	96
TNF-α	*Tumor necrosis factor alpha*	EU682384.1	Ss03391318_g1	73
IL-10	*Interleukin-10*	AK397697.1	Ss03382372_u1	87
NLRP3	*NLR family pyrin domain containing 3*	AB292177.1	Ss04953522_m1	109
CCL2	*Chemokine (C-C motif) ligand 2*	EU682382.1	Ss03394377_m1	58
CXCL8	*C-X-C motif chemokine ligand 8*	AK231005.1	Ss03392437_m1	94
HPRT1	*Hypoxanthine phosphoribosyltransferase 1*	NM_001032376.2	Ss03388274_m1	73

RT-qPCR, real time quantitative PCR.

2.5 Statistical Analysis

Data treatment was performed using Statistical Analysis Software, version 9.4 (SAS Institute, Cary, NC, USA) [39]. All data are presented as means with their standard errors. Data were checked for normal distribution by Shapiro–Wilk test and variance homogeneity by Levene's test. Data were analyzed by using Proc MIXED with a model including tissue and dietary treatment as fixed effects and the repeated statement considering the group option to accommodate the variance heterogeneity. Statistical differences among tissues and dietary treatments were evaluated by least square means generated using the PDIFF option adjusted with Tukey–Kramer. Pearson's correlation coefficients were performed with the Proc CORR procedure to establish linear relationships among gene expressions. A principal component analysis (PCA) was performed with AQPs and cytokines gene expression levels from all tissues: ileum, liver, spleen, and kidney. The PRIN COMP procedure was applied to 108 samples and 14 variables to diminish the dimensionality of the data set and to describe the variability of data into two dimensions. After data normalization, the principal components were interpreted as significant if they contributed more than 5% of the total variance. All statistical tests were considered significant at a probability level of 5%.

3. Results

3.1 Aquaporins Transcriptional Profile is Tissue-Specific but not Dependent on Lipopolysaccharide or Dietary Treatment

Fig. 1A–G show the variations on gene expression levels of AQPs across tissues from piglets under LPS challenge, with or without dietary amino acids (arginine, BCAA and cystine combined) supplementation. AQPs transcriptional profiles were tissue-specific ($p < 0.001$), as described next. No effects by the diets or LPS were found for *AQPs* ($p > 0.05$). *AQP1* was highly expressed in the kidney, intermediate in the ileum and spleen, and minimally expressed in the liver (Fig. 1A). *AQP2* was only expressed in the kidney (Fig. 1B). The *AQP3* mRNA levels were higher in the small intestine and kidney, intermediate in the spleen, and lowest in the liver (Fig. 1C). *AQP5* was undetected in the liver and spleen, minimally expressed in the kidney, and reached the highest level of expression in the small intestine (Fig. 1D). *AQP8* reached the highest levels of mRNA expression in the ileum, was minimally expressed in the liver, and was undetected in the spleen and kidney (Fig. 1E). *AQP9* was mostly expressed in the liver, intermediately in the ileum, and residually in the kidney and spleen (Fig. 1F). Finally, *AQP10* was only expressed in the ileum and was undetected in the liver, spleen, and kidneys (Fig. 1G).

3.2 Cytokines Transcriptional Profile is Tissue-Specific but not Dependent on Lipopolysaccharide or Dietary Treatment

Fig. 2A–G show the variations in gene expression levels of cytokines across tissues from piglets under LPS challenge, with or without dietary amino acid (arginine, BCAA, and cystine combined) supplementation. The inflammation was highly dependent on tissue ($p < 0.001$), as described next. *CCL2* reached the highest levels of expression in the ileum, followed by the spleen and kidney, with minimal levels of expression in the liver (Fig. 2A). Ileum showed the highest mRNA levels of *CXCL8* and the lowest in the kidney, spleen, and liver (Fig. 2B). Similarly, *IL-6* was highly expressed in the ileum, followed by the kidney and spleen, and lastly in the liver (Fig. 2D). *TNF-α* was mostly expressed in the ileum, reasonably expressed in the liver and kidney, and minimally in the spleen (Fig. 2E). *NLRP3* reached the highest mRNA levels in the ileum, followed by

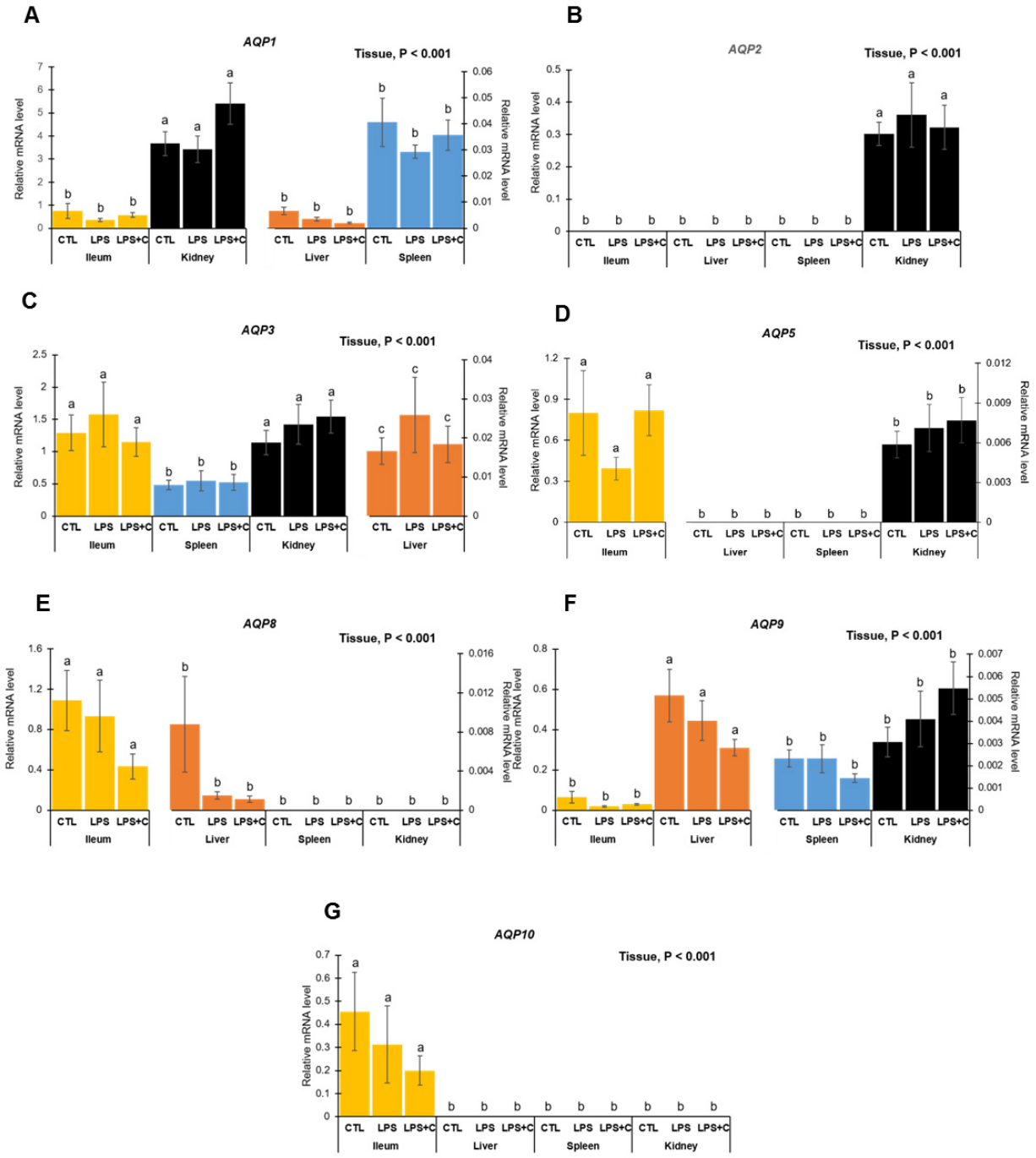

Fig. 1. Effect of tissue, dietary treatment, and tissue with dietary treatment interaction on the relative gene expression levels of *AQP1* (A), *AQP2* (B), *AQP3* (C), *AQP5* (D), *AQP8* (E), *AQP9* (F), and *AQP10* (G) in the ileum (yellow bars), liver (orange bars), spleen (blue bars), and kidney (black bars) from piglets fed with control (CTL), under LPS challenge (LPS), and under LPS challenge with dietary amino acid (arginine, BCAA (leucine, valine, and isoleucine), and cystine combined) supplementation (LPS + C). Values are presented as mean with standard errors represented by vertical bars. ^{a,b,c} mean values with unlike letters were significantly different (Tukey's *post hoc*, $p < 0.05$).

the kidney, then, the spleen, and lastly, the liver (Fig. 2F). *IL-10* presented a similar pattern of variation being more prevalent in the ileum, then, the spleen and kidney, and ultimately, in the liver (Fig. 2G).

3.3 IL-1β is Affected by the Interaction between Tissue and Dietary Treatment

The only cytokine with a different pattern of gene variation across tissues from piglets under LPS challenge, with or without dietary amino acids (arginine, BCAA, and cys-

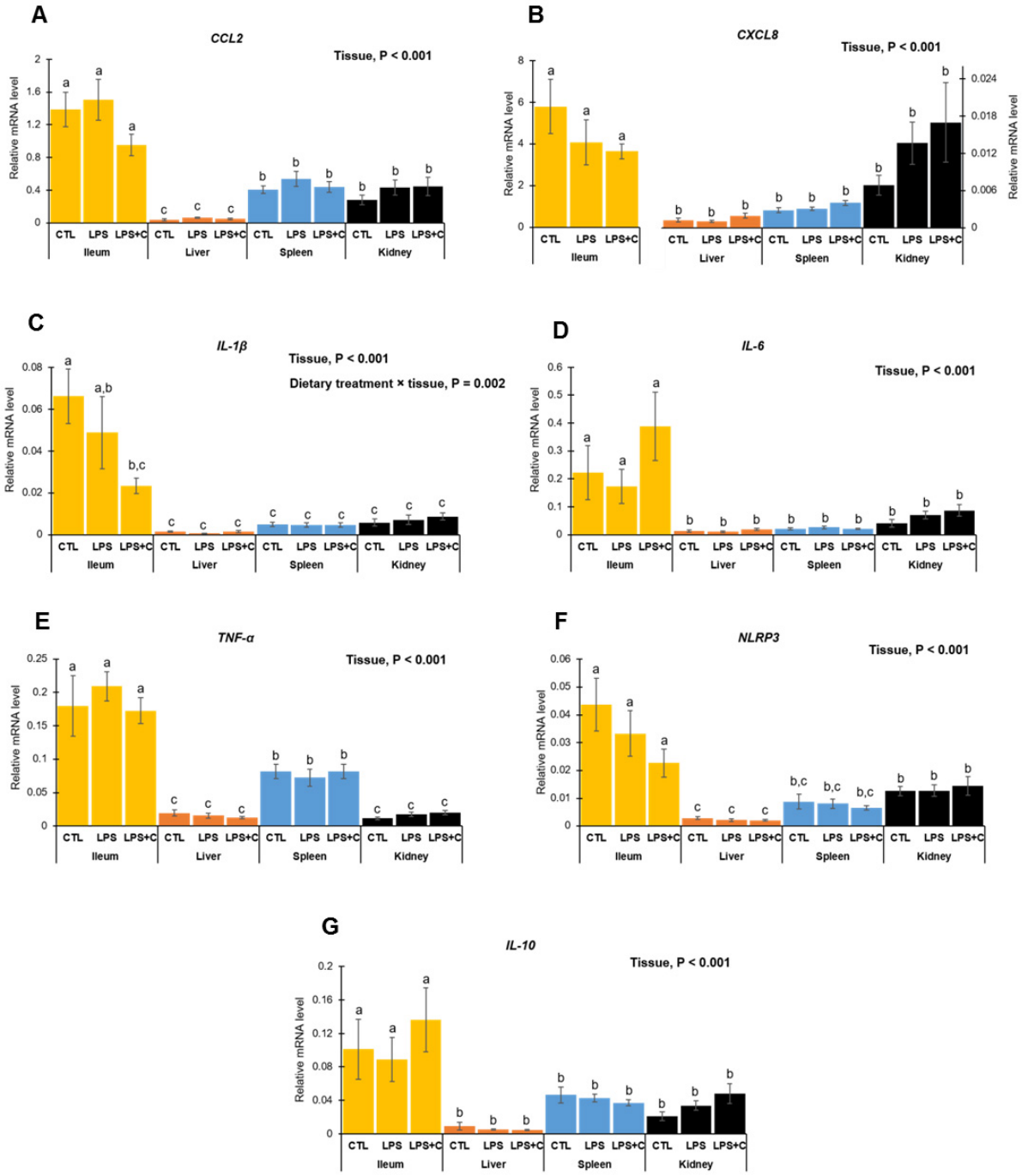

Fig. 2. Effect of tissue, dietary treatment, and tissue with dietary treatment interaction on the relative gene expression levels of *CCL2* (A), *CXCL8* (B), *IL-1β* (C), *IL-6* (D), *TNF-α* (E), *NLRP3* (F), and *IL-10* (G) in the ileum (yellow bars), liver (orange bars), spleen (blue bars), and kidneys (black bars) from piglets fed with control (CTL), under LPS challenge (LPS), and under LPS challenge with dietary amino acids (arginine, BCAA (leucine, valine, and isoleucine), and cystine combined) supplementation (LPS + C). Values are presented as mean with standard errors represented by vertical bars. ^{a,b,c} mean values with unlike letters were significantly different (Tukey's *post hoc*, $p < 0.05$).

tine combined) supplementation was *IL-1β* (Fig. 2C). In terms of magnitude, it was mostly expressed in the ileum of the small intestine of piglets followed by the kidney, then, the spleen, and lastly, in the liver. *IL-1β* was affected by the interaction between tissue and dietary treatment, whereby it was reduced in piglets under LPS and fed the amino acid cocktail diet relative to the control in the ileum ($p = 0.035$) (Fig. 2C). The same was not observed for the other tissues ($p > 0.05$).

3.4 Pearson Correlation Coefficients among mRNA Expression Levels of Aquaporins and Cytokines per Tissue

Fig. 3 presents Pearson's correlations coefficients among mRNA expression levels of AQPs and cytokines in the ileum (Fig. 3A), liver (Fig. 3B), spleen (Fig. 3C), and kidney (Fig. 3D) of piglets under LPS challenge, with or without dietary amino acid (arginine, BCAA and cystine combined) supplementation. All the correlations were found to be positive. Since the number of correlations were very high per tissue, only the highest correlations are herein described (high correlation, $r > 0.7$; moderate correlation, $0.7 \geq r \geq 0.3$; low correlation, $r < 0.3$) [40].

In the ileum (Fig. 3A), *AQP1* correlated to *AQP5* ($r = 0.773$, $p < 0.001$) and *AQP9* ($r = 0.903$, $p < 0.001$). *AQP5* and *AQP9* correlated with each other ($r = 0.751$, $p < 0.001$). *CXCL8* correlated to *IL-1*β ($r = 0.823$, $p < 0.001$). *CCL2* correlated to *TNF-*α ($r = 0.740$, $p < 0.001$). Finally, *IL-6* was found to be strongly correlated with *IL-10* ($r = 0.979$, $p < 0.001$).

In the liver (Fig. 3B), *AQP1* correlated to *NLRP3* ($r = 0.709$, $p < 0.001$). *AQP9* correlated to *TNF-*α ($r = 0.840$, $p < 0.001$) and *IL-10* ($r = 0.785$, $p < 0.001$). Finally, *TNF-*α was strongly correlated to *NLRP3* ($r = 0.801$, $p < 0.001$) and *IL-10* ($r = 0.729$, $p < 0.001$).

In the spleen (Fig. 3C), *AQP1* correlated to *IL-1*β ($r = 0.755$, $p < 0.001$), while being strongly correlated to *IL-10* ($r = 0.840$, $p < 0.001$). *IL-6* correlated to *TNF-*α ($r = 0.749$, $p < 0.001$) and *CCL2* ($r = 0.701$, $p < 0.001$). Finally, *IL-10* correlated to *IL-1*β ($r = 0.737$, $p < 0.001$) and *NLRP3* ($r = 0.725$, $p < 0.001$).

In the kidneys (Fig. 3D), *AQP1* correlated to *AQP3* ($r = 0.782$, $p < 0.001$). *AQP2* correlated to *AQP3* ($r = 0.779$, $p < 0.001$) and *AQP5* ($r = 0.786$, $p < 0.001$). *AQP3* and *AQP5* were correlated to each other ($r = 0.716$, $p < 0.001$). *TNF-*α correlated to *AQP2* ($r = 0.710$, $p < 0.001$), *AQP3* ($r = 0.772$, $p < 0.001$) and *AQP5* ($r = 0.725$, $p < 0.001$). *CCL2* was strongly correlated to both *CXCL8* ($r = 0.876$, $p < 0.001$) and *IL-1*β ($r = 0.736$, $p < 0.001$). *IL-6* correlated to *NLRP3* ($r = 0.723$, $p < 0.001$) and was strongly correlated to *IL-10* ($r = 0.923$, $p < 0.001$). Finally, *NLRP3* and *IL-10* correlated with each other ($r = 0.777$, $p < 0.001$).

3.5 Principal Component Analysis

Fig. 4 shows the principal component analysis (PCA) output applied to the data set of 27 animal samples with 14 variables per tissue: ileum, liver, spleen, and kidney. The first and second principal components accounted for 64.14% of the total variance, with 46.61% for component 1 and 17.53% for component 2. As the total variance, explained by the first two principal components, was higher than 50%, the projection of animal tissue samples in the plane defined by these components is illustrated in Fig. 4.

A differential pattern of AQPs gene expression and cytokines across the ileum, liver, spleen, and kidneys was clearly observed. It set apart the ileum located across quadrants (b) and (c) and the kidney located in the upper part of the graphic from the liver and the spleen that appeared mostly in quadrant (d). In view of the fact that the liver and spleen are positioned very close to each other, we suggest the existence of a similar tissue-specific pattern for AQPs and cytokines gene expression, regardless of the LPS intraperitoneal injection or the amino acid mixture supplementation. Table 3 displays the loadings for the first two principal components. Overall, component 1 was mainly characterized by cytokines gene expression data, in particular *CCL2* (0.340), *TNF-*α (0.340), *IL-1*β (0.333), *CXCL8* (0.331), and *NLRP3* (0.322), while component 2 was mainly characterized by AQPs gene expression data, in particular *AQP1* (0.599), *AQP2* (0.599), *AQP3* (0.391), and *AQP9* (–0.277).

Table 3. Loadings for the first two principal components of principal component analysis (PCA).

	Eigenvectors	
	Component 1	Component 2
AQP1	0.013	0.599
AQP2	–0.042	0.599
AQP3	0.260	0.391
AQP5	0.314	–0.072
AQP8	0.262	–0.072
AQP9	–0.106	–0.277
AQP10	0.243	–0.062
CCL2	0.340	0.014
CXCL8	0.331	–0.113
IL-1β	0.333	–0.049
IL-6	0.248	0.012
TNF-a	0.340	–0.127
NLRP3	0.322	0.080
IL-10	0.275	0.023

4. Discussion

Herein, we provided a screening of AQPs and cytokine gene expressions, across the ileum, liver, spleen, and kidneys, from piglets under a proinflammatory environment. Contrarily to the literature, using different experimental models and LPS dosages [2,3,14,18,27], no major impact of LPS injection on different AQP isoforms and cytokine transcriptional profiles was found. This is intriguing and most probably related to the chronological differences between the intraperitoneal injection of LPS and the piglets' euthanasia (a time period of 5 weeks), which potentially provided enough time for animals' immune recovery. We believe that to achieve statistical differences, LPS-challenged piglets should have been sacrificed 24 h after LPS [35]. The LPS animal model has many advantages, including technical ease and good reproducibility, especially in the elicited inflammatory response [3].

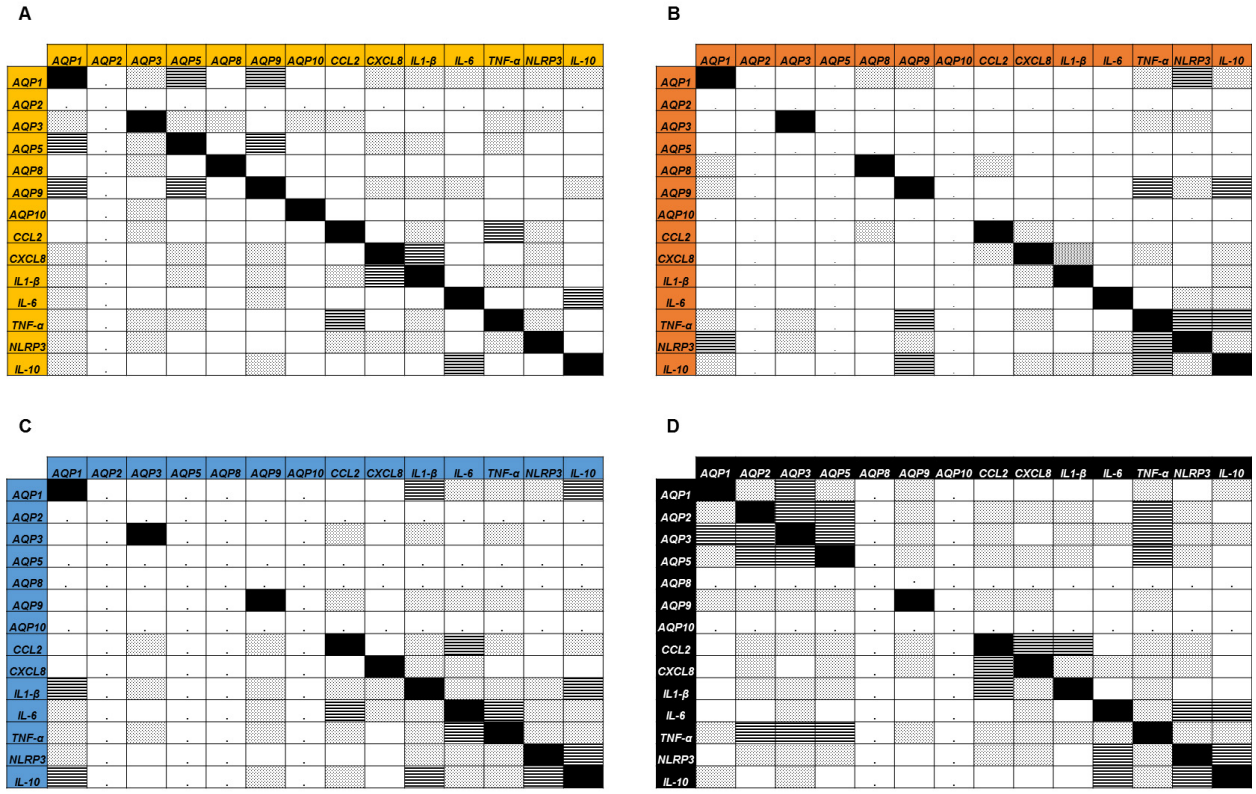

Fig. 3. Pearson's correlation coefficients among mRNA expression levels of AQPs and cytokines in the ileum (yellow, panel A), liver (orange, panel B), spleen (blue, panel C), and kidneys (black, panel D) from piglets fed with control, under LPS challenge, and under LPS challenge with dietary amino acids (arginine, BCAA (leucine, valine, and isoleucine), and cystine combined) supplementation. White squares, NS; black dots squares, $p < 0.01$; black stripes squares, $p < 0.001$.

In this study, half of the LPS-challenged piglets were fed a diet supplemented with a cocktail of functional amino acids. The dietary amino acid mixture did not reverse the negative influence of LPS intraperitoneal injection over AQPs and cytokines gene expression levels in piglets, as initially hypothesized, because only a minor variation was detected for IL-1β across tissues, which is devoid of any clinical physiological relevance. In view of our results, the potential molecular mechanisms that could mediate the effects of dietary supplementation with amino acids - arginine, BCAA (leucine, valine, and isoleucine), and cysteine - on the LPS-induced fluxes in aquaporins and proinflammatory cytokines gene expressions remain elusive.

Moreover, we postulated that AQPs and pro- and anti-inflammatory markers display their own specific gene expression patterns, according to the target tissue. This is a major breakthrough since, to the best of our knowledge, the full characterizations of the AQPs transcriptional profiles have not previously been carried out, especially for the gastrointestinal tract (*e.g.*, [9,27,41–46]). Notwithstanding, the mRNA levels of the AQPs detected per tissue are in accordance with several available reports in the literature, in particular for *AQP3* and *AQP10* in the small intestine [47,48], *AQP1*, *AQP3*, *AQP8*, and *AQP9* in the liver [49–51], *AQP3* and *AQP9* in the spleen [52], and *AQP1*, *AQP2*, and *AQP3* in the kidney [47,53].

The tissue-specific signature for AQPs and cytokine gene expressions was corroborated by a discriminant analysis tool that clearly distinguished the ileum and the kidney from the liver and spleen; these last organs are more identical to each other. We also describe a strong interplay among AQPs, according to tissue, which was sustained by high positive Pearson's correlations. A high positive correlation is a large positive relationship since the value is close to +1 [39], meaning that both variables move in the same direction. This statistical tool supports the tissue-specific operating mode of aquaporins, for both water and glycerol permeation [8].

In what concerns to inflammation, serum TNF-α was unaffected by the LPS challenge, as previously reported by Prates *et al.* [35], in agreement with non-variations on the corresponding transcriptional profile in the ileum, liver, spleen, and kidney. In this regard, Remick *et al.* [54] described that shortly after LPS administration, high levels of proinflammatory cytokines were secreted and could be quantified in serum. Webel *et al.* [55] and Moya *et al.* [56] reported that intraperitoneal injection of LPS was associated with a transient increase in serum TNF-α. NLRP3 inflammasome activation was also confirmed in LPS-induced DLB mice [57]. In THP1 human monocytic cells, AQP3

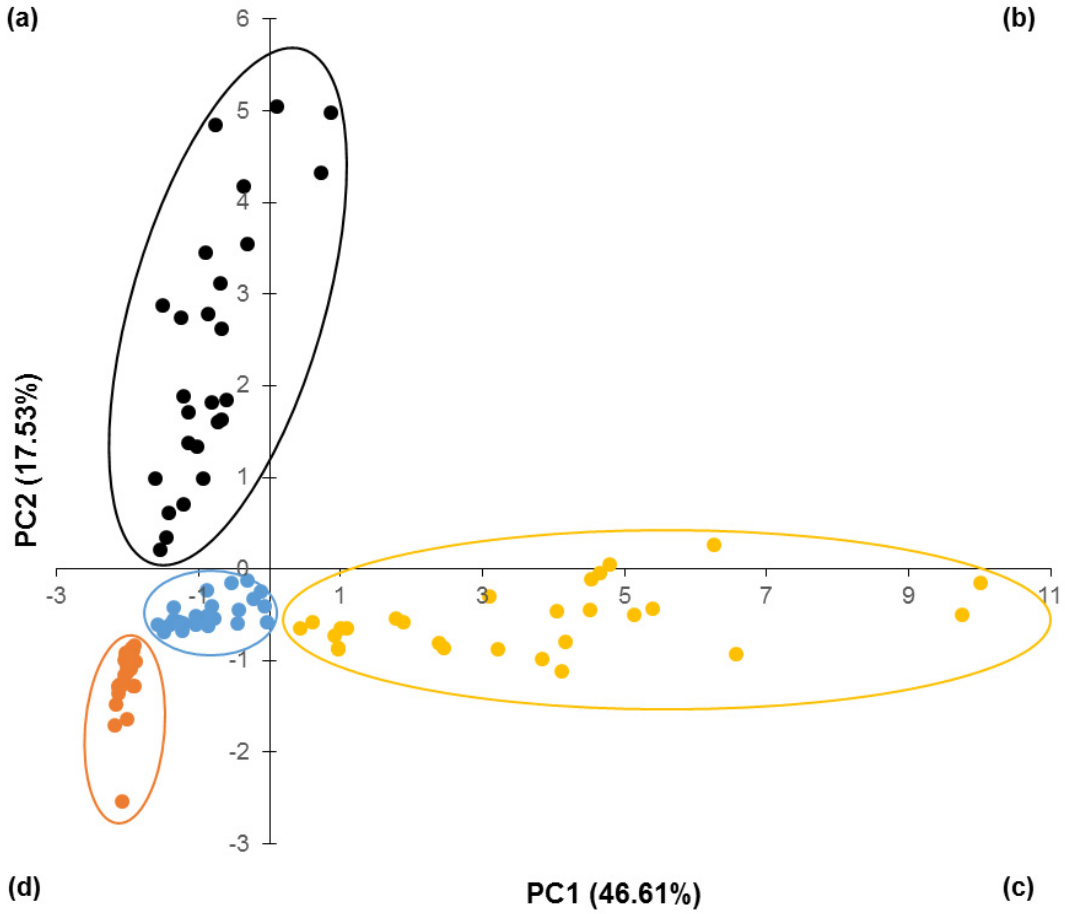

Fig. 4. Projection of samples (average of AQPs and cytokines gene expression levels by tissue: ileum (yellow), liver (orange), spleen (blue), and kidneys (black)) from piglets fed with control, under LPS challenge, and under LPS challenge with dietary amino acids (arginine, BCAA (leucine, valine, and isoleucine), and cystine combined) supplementation, in the plane defined by the two first principal components of principal component analysis (PCA).

was involved in LPS priming by Toll-like receptor 4 engagement, which results in NLRP3 and IL-6 upregulation [58]. In fact, it is well established that AQPs have a crucial role in the settings of inflammation with implications on inflammatory diseases and sepsis [8]. Reinforcing these findings, the LPS group exhibited increased haptoglobin and cortisol levels on day 10 after LPS intraperitoneal injection, as reported by a companion paper [35]. Immunoglobulin concentrations were also affected by dietary treatments [35]. In fact, on day 10 after LPS, the concentrations of IgG and IgM were reduced by LPS, although at the time of euthanasia, the immunoglobulin concentrations did not vary between the LPS and control groups [35]. Moreover, the proteomics analysis did not reveal relevant alterations of the protein profile in response to the LPS challenge or dietary amino acid supplementation [35]. In addition, IL-1β presented no changes in the serum (CTR = 28.4; LPS = 19.4; LPS + cocktail = 20.3 pg/mL; SEM = 6.63; $p = 0.719$), while IL-6 was found below the minimum detectable dose (MDD, <2.03 pg/mL), pointing towards the non-existence of inflammation at the time of euthanasia. These findings are in line with results found for interleukin mRNA levels. Among the statistical correlations found, it should be underlined that IL-6 and IL-10 gene expression were positively correlated in all tissues studied. Considering that IL-6 is accepted as a proinflammatory cytokine, whereas IL-10 is anti-inflammatory [59], these variations ascertain how the trigger of inflammation is physiologically accompanied by an anti-inflammatory response. The same rationale applies to TNF-α and IL-10 in the liver, spleen, and kidneys.

According to the literature, pigs are very sensitive to the administration of small dosages of LPS, which cause an immediate intense inflammatory response without causing mortality [60,61]. We advance these findings to demonstrate that this immediate but profound inflammatory response disappears as time goes by.

Two important limitations of this study should be acknowledged. Gene expression changes often exhibit no or probably nonlinear correlation with protein expression levels, and unfortunately, protein level changes in the AQPs and cytokines were not assessed in this study. Since gene expression changes temporally precede the protein expres-

sion changes, we hypothesize that a later or subsequent endpoint analysis may exhibit changes in protein expression levels that are currently not reflected at the chosen moment of the endpoint analysis. This remains to be further elucidated. Another important limitation is that the number of piglets included in this experimental assay, in particular in the LPS-challenged group without amino acid supplementation, was relatively small. Therefore, large-scale studies are necessary to investigate if the modulation over inflammation can be translated into relevant effects on the animal's performance and even survival.

Notably, the current work points towards the unveiling of novel strategies to target these channels. Targeting the molecular and signaling mechanisms of AQPs, in addition to using AQP-targeted antibodies, microRNAs, or other biomolecules, has been an experimental strategy to evaluate novel pharmacological modulators that directly affect aquaporin function, uncovering new therapeutic perspectives [7,11,62,63]. Furthermore, the screening of AQPs as transport modulators, diagnostic clinical biomarkers, and even drug targets in a porcine model might be successfully translated to humans [36,61].

5. Conclusions

The systematic methodological approach herein presented for AQPs and cytokines, based on gene expression profiles, in some key tissues of the gastrointestinal tract that control sepsis, enables the elucidation of tissue-specific variations for these transmembrane proteins and inflammatory mediators, thereby providing a novel insight into their operating physiological modes of action, *i.e.*, across the small intestine, liver, spleen, and kidney, by using a porcine model of inflammation. Since the ileum and the kidney were found to be clearly separated from the other tissues, by a discriminant analysis tool, we propose the existence of a similar tissue-specific pattern for AQPs and cytokines gene expression, regardless of LPS intraperitoneal injection. No major variations were detected in the mRNA levels of the AQPs and inflammatory mediators, upon the dependence on LPS or the amino acid cocktail supplementation, suggesting an immune recovery by the piglets. Further *in vivo* investigation based on optimized LPS dosages and recovery time is of the utmost requirement to clarify the role of AQPs and cytokines in relation to inflammation.

Availability of Data and Materials

The data presented in this study are available in this article.

Author Contributions

IVS: Conception of the work, acquision and analysis of data; DC: analysis and interpretation of data; JAMP: conception and design of the work; GS: conception of the work, writing - reviewing and editing; PAL: conception of the work, writing - original draft preparation, writing - reviewing and editing. All authors substantially contributed to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work and drafted or critically revised the work for important intellectual content and approved the final version to be published and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The experimental procedures of the animal trial were carefully reviewed and approved by the Animal Care Committee of Instituto Superior de Agronomia (ISA), Universidade de Lisboa, (Lisbon, Portugal) and authorized by the National Veterinary Authority (Direcção Geral de Alimentação e Veterinária (Lisbon, Portugal), according to European Union guidelines (2010/63/EU Directive). The ethical approval code is #0421/000/000/2017. JAMP and PAL hold a FELASA grade C certificate, and DC hold a FELASA grade B certificate, which enables them to conduct animal experimentation in the European Union.

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Conflict of Interest

The authors declare no conflict of interest.

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