

Review

How Is the Effect of Phytogenic Feed Supplementation Tested in Heat Stressed Pigs? Methodological and Sampling Considerations

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Abstract: Climate change will lead to increasingly hot summers where the temperature rises above the thermoneutral range of pigs; as a result, they get exposed to heat stress. One of the most damaging consequences of long-lasting heat stress is oxidative stress arising from the increasing level of reactive oxygen species. In order to eliminate oxidative stress, metabolites that are needed for maintaining life and growth may get depleted, which, in chronic cases in particular, negatively affects the economy of meat production. The effect of plant-originated phytogenic feed additives with high antioxidant content may be beneficial to pigs in reducing the effects of oxidative stress induced by heat stress. In this study, a range of methods that assess the effects of phytogenic feed additives on heat stress are reviewed. The main focus is presenting an overview of the investigational possibilities of the antioxidative system and feed uptake and utilization via traditional methods and molecular biological investigations. Furthermore, methodological aspects of sampling are taken into consideration in order to select the best methods for determining the effect of phytogenic feed supplementation on heat-stressed pigs.

Keywords: heat stress; antioxidants; molecular methods; gene expression; pig; phytogenic feed supplementation

1. Introduction

Climate change poses a major burden to agriculture, especially because of the extreme weather it causes, frequently manifested in alterations in water supply, or excessively hot or cold conditions. Heat stress (HS) poses challenges to both crop production and animal husbandry. In animal husbandry, HS is a major factor as it affects the physiology, metabolism and behavior of animals. The production of pork takes place mainly in areas where the temperature of the environment is periodically over the thermoneutral range of pigs, and there is a severe fluctuation in day temperature to which the animals have to adapt [1]. As a result, the risk of HS is more frequent and severe in pork production than ever before.

In order to maintain homeostasis, the body temperature of pigs should stay within a relatively narrow range (38–39 °C). To release the heat into the environment, the peripheral blood flow increases [2]

in order to maintain a normal intestinal temperature. However, this reduces the oxygen supply to the digestive system, which may lead to nutrient deficiency. Consequently, HS negatively affects the performance and intestinal integrity of pigs [3–5]. Pearce et al. [6], who examined parameters of intestinal integrity and metabolism during acute heat-stressed pigs, found that an increase in ileum heat shock protein 70 expression as an indicator of oxidative stress. Furthermore, they reported that HS decreased feed intake by 53% and compromised intestinal integrity. The serum endotoxin level was also found to be increased. Myosin light chain kinase, casein kinase II-a, tight junction (TJ) proteins, claudin 3 and occludin expression increased due to HS. According to Pearce et al. [6], the increased expression of claudin and occludin is due to an increase in intestinal permeability due to HS, when the animal metabolism attempts to compensate for this with increased synthesis of proteins that promote tight junction. Intestinal glucose transport and blood glucose were elevated due to HS, as were ileum Na^+/K^+ ATPase activity and ileal glutamine transporter 2 protein (GLUT-2) expression [6].

As a result, the proliferation of intestinal cells decreases [2], and reactive oxygen species (ROS) are formed in the muscles that may accumulate, initiating oxidative stress [3]. HS induces oxidative stress, which occurs when the amount of ROS increases to such an extent that the defense mechanisms do not prove to be sufficient to eliminate them. ROS can damage vital macromolecules: lipids, proteins and nucleic acids [6]. The elimination of stress-induced ROS strains the metabolism of pigs, similar to in other animals, and depletes the metabolite pools required for the homeostasis of normal life and growth. All these initiate a negative economic impact, causing a decrease in the amount of meat and a deterioration in the meat quality that needs to be dealt with.

In addition to optimizing housing conditions, feeding is an area in which the physiological condition of pigs can be improved. The innovation in nutritional sciences proceeds from the addition of synthetic additives along with natural extracts and additives. Extracts from plants containing biologically active substances are of interest for their antioxidant and anti-inflammatory effects [7,8]. Antiviral and antimicrobial activity was found by Bakht et al. [9], the mode of action of which is deteriorating the glycolipid walls of bacterial cells, causing the outflow of the cytoplasm, as reported by Iranparast et al. [10]. Due to their high antioxidant content, the use of plant-based phytogenic feed additives (PFAs) or complementary feeds may help to prevent or ameliorate the effects of HS [9–12].

The aims of this work are to provide an overview of the current literature about the effects of HS in pigs, and to elucidate the possible applications of PFAs in order to reduce the detrimental effects of HS in pig production. The focus is on the results of physiological and molecular biological research regarding the thermal stress-reducing effects of feed additives of phytogenic origin.

2. Formation and Consequences of HS Induced Oxidative Stress in Pigs

One of the most important abiotic stress agent in animal husbandry and feeding is HS, which occurs when the ambient temperature rises above 25 °C. As a consequence, cardiac function accelerates and peripheral circulation increases [2], but voluntary feed intake decreases [7,13].

According to Huynh et al. [13], the development of the effects of HS shows the following sequence: first, in the range of 21.3 to 23.4 °C, breathing intensity increases, followed by a decrease in water and feed intake ratios. Thereafter, between 22.9 and 25.5 °C, heat production and feed intake are reduced, and then, finally, the intestinal temperature hits 24.6 to 27.1 °C.

These processes are related to oxygen utilization. Oxygen is the ultimate electron acceptor of the respiratory chain. However, during metabolic processes, partially reduced oxygen forms are produced, which are highly reactive, superoxide and hydroxyl radicals that have an unpaired electron, or molecules that are capable of forming free radicals in their reactions, e.g., hydrogen peroxide and singlet oxygen are produced [14]. In summary, these radicals and compounds are called ROS or prooxidants. ROS are constantly produced in cells during metabolic processes, regardless of whether the animal is stressed or not. A well-organized defense mechanisms exist in animals to neutralize ROS, ideally, the formation and binding of oxygen radicals is in equilibrium [15,16].

In the case of HS-induced oxidative stress, this equilibrium may shift towards the accumulation of ROS that exceeds the antioxidant capacity. In this case, ROS may damage vital macromolecules: proteins, nucleic acids and lipids [7]. The deterioration of the latter manifests in oxidative processes resulting in unstable lipid peroxides that tend to rapidly degrade to various sub-products. In this context, the integrity of cellular organs may also be disrupted, which cause metabolic imbalances as well as DNA damage that leads to gene expression disturbances, along with functional disorders resulting in diseases and mutations [17]. The evolution of oxidative stress depends on the intensity of ROS production and on the overall capacity of the antioxidant system. If the HS is long-lasting, the disruption of homeostasis may occur when the animal uses up its original antioxidant capacity and harmful metabolic processes are initiated. These processes have a negative impact on the quantity and quality of meat, and consequently on the economy [14,18–21]. In order to eliminate these detrimental processes, the constitutively present antioxidants need to be investigated and their amount should be determined.

2.1. Effect of HS on the Antioxidative System of Pigs

There are several methods for quantifying antioxidant compounds, among which total antioxidant content may be the most important, since it plays a key role in neutralizing ROS triggered by HS. Thus, in the interest of gaining a clear picture on the possible HS-eliminating capability of PFAs, it is worth determining the total free radical scavenging capacity.

The determination of the total radical scavenging capacity of different tissues shows the combined radical scavenging capacity of the non-enzymatic antioxidant compounds present in the sample. Different methods are available for total antioxidant capacity determination, because of the complex and far-branching nature of the antioxidant system and the specificity of the methods [22–31]. Malondialdehyde (MDA) can also be used as a marker of cell membrane damage resulting from lipid peroxidation, so it is a good indicator of the development of oxidative stress caused by HS [32]. The total radical scavenging capacity and lipid oxidation can be measured in a number of biological samples [33], such as blood plasma, blood serum, different tissues and occasionally in the urine. Since PFAs are rich in antioxidants, they may positively alter the antioxidant capacity [34–36] and peroxidation state, which is why their measurement might provide useful information on the overall antioxidant status of different tissues [35].

The other major part of the antioxidant system is based on enzymatic antioxidants. The antioxidative enzyme system consists of several enzymes, such as superoxide dismutase (SOD) [37–39], glutathione peroxidase (GPX) and catalase (CAT) [40], methionine disulfide reductase [41], thioredoxin 5'-dejodinase [42], glutathione reductase-GR and glutathione-S-transferase-GST [39], which are responsible for the elimination of ROS in the different cellular compartments. Their changes may also be informative in terms of to what extent the feed dose or the supplementation contributes to a reduction in HS-induced oxidative stress, or whether a feed intake increase induced stress resistance of the tested genotypes.

Yang et al. [43] investigated the effect of HS on pigs and tested the meat quality, malondialdehyde (MDA) content, CAT and SOD activities and carnosine synthetase (*CARNS1*) mRNA expression in the *longissimus dorsi* muscle after three weeks of HS (Table 1). They found that HS affected meat quality and antioxidant capacity in a detrimental way: a higher MDA level and lower SOD and CAT activity were found in the *longissimus dorsi* muscle of pigs that were kept at 30 °C. Similarly, Chen et al. [32] found an increased MDA concentration and decreased SOD activity after 48 h of oxidative stress in porcine kidney cells. Lopez et al. [35] observed that pigs kept at high temperatures (22.5–35 °C) grew 16.3% more slowly and consumed 10.9% less feed than their peers kept at constant 20 °C conditions. Liu et al. [29] investigated the effects of HS on oxidative status in growing pigs and determined different antioxidant parameters (biological antioxidant potential, oxy-absorbent capacity and thiols), as well as an oxidative damage marker—advanced oxidized protein—products and were able to trace the effects of HS-induced oxidative stress [36]. Cui et al. [43] compared the oxidative stress response,

of finishing-stage pig liver during HS and found that the concentrations of GSH and GSSG GST and GPX enzymatic activity were elevated by 21 days of HS, whereas the GSH/GSSG ratio decreased, indicating that the antioxidant pathway may also be influenced by HS interfering with the oxidative state of liver tissue. They concluded that the GSH/GSSG ratio was an indicator of oxidative stress [43]. Crossbred gilts were assigned to temporary (2, 4, 6 h) HS (37 °C) and they measured antioxidant parameters from the red portion of the semitendinosus muscle. The concentration of MDA increased after 2 and 4 h of HS, as did the catalase activity. They concluded that HS caused a transient increase in oxidative stress, which the increase in catalase activity attempted to compensate for [44] (Table 1).

Table 1. Sampling for heat stress (HS)-related investigation of the antioxidative system and metabolites.

| Frequency of Sampling | Weight of Animals (kg) | Conditions of Slaughter | Investigated Parameter | Sample Type | Age of Animals/Duration of Experiment (Days) | Ref. |
|-----------------------------|------------------------------|--|---|--|--|------|
| at slaughter | 50 ± 2 | 3% intravenous sodium pentobarbital | Creatine-kinase, aspartate-aminotransferase | blood serum | 120/0 | [37] |
| at slaughter | 63.8 ± 2.9 | bleeding after electric stunning | MDH, antioxidant capacity, antioxidative enzymes, cortisol, noradrenaline, lipid peroxidation, antioxidant capacity | liver blood serum | 120/28 | [43] |
| 3, 7, 14, 21 days | 40.8 ± 2.7 | electric stunning | insulin, IGF-1, IGFBP-1, IGFBP-3, glucose concentration | blood serum | 80/22 | [45] |
| at slaughter | 79 ± 1.5 | bleeding after electric stunning | MDA, CAT, SOD | longissimus dorsi muscle | data not available/21 | [41] |
| 1st, 7th days of experiment | 28 ± 3 | data not available | biological antioxidant potential, oxy-absorbent capacity, thiols, erythrocyte GPx, oxidative damage marker | blood, erythrocyte, | data not available/7 | [36] |
| at slaughter | 79 ± 1.5 | head only electric stun tong apparatus | GPx, GSH/GSSG, GST | liver | data not available/21 | [42] |
| 1, 3, 6, 10 days | 7.15 ± 0.58 | anesthesia | glucose level | serum | 60/10 | [46] |
| 0, 42, 84 and 126 days | 24.7 ± 0.27 | - | Cortisol, norepinephrine, epinephrine Dietary crude protein, phosphorus, nitrogen, chromium | blood feces | data not available/1–18 weeks | [15] |
| at slaughter | 16.31 ± 1.26 29.55 ± 1.27 | electrical stunning | SOD, CAT, GPx, total antioxidant capacity TAOC | Longissimus thoracis muscle | 50/95 | [42] |
| at slaughter | 63.8 ± 2.9 | pentobarbital injection | MDA, protein carbonyl content, CAT, SOD | red portion of the semitendinosus muscle | 2, 4, 65 h | [43] |
| at slaughter | 114 ± 2 | data not available | SOD, MDA | longissimus dorsi muscle, backfat, liver | 6, 9, 12 months | [30] |
| at slaughter | 7.10 ± 0.52 | head-only electric stun tong | cortisol | serum | 60/1,6 | [47] |
| at slaughter | 77.80 ± 4.25 | electrical stunning | SOD, GSH-Px, TAOC | serum, liver | data not available/28 | [33] |

2.2. Effect of HS on Related Gene Expression in Pigs

Stressors alter existing metabolic pathways directly, and may induce the over- or underexpression of certain genes that initiate alternative biochemical processes that have not worked before. Metabolic changes due to HS are often the result of gene expression changes; therefore, the target genes can be used as stress indicators, and the changes in expression are more likely to provide earlier information

on stress status and its extent than the investigations of metabolic parameters. There are several examples in the related literature on using gene expression studies for detecting and investigating the consequences of HS.

In pigs, the first signs of HS are the induction of heat shock proteins (HSPs), the main function of which is to restore the correct spatial structure of proteins that have lost their conformation. Gene expression studies of pigs kept at controlled temperature and subjected to HS by daily heat fluctuations showed an increase in expression of the *HSP90* gene [1]. It was found that the expression of heat shock proteins was uniformly increased in stressed animals.

Pearce et al. [5] found that in animals with an average weight of 24.64 ± 3 kg and ad libitum feeding, after 12 h of 37°C HS, the expression of small intestine *HSP27*, *HSP70*, *HSP90* and pyruvate dehydrogenase kinase (*PDK4*) genes increased.

Du et al. [30] examined the differences in the expression of the *CuZn-SOD* enzyme gene in the liver, muscle and adipose tissue. They did this because the genotype determines the animal's original antioxidant abilities, which can be modified by environmental effects. Different levels of gene expressions have been identified in each organ and between genotypes and it has been concluded that the activity of the *CuZn-SOD* enzyme has the effect of maintaining the antioxidative balance of meat and meat quality.

In addition to the antioxidant pathways to prevent stress, metabolic pathways may also change when the organism mobilizes its energy reserves to overcome stress. In these processes, typical transport compounds, such as amino acids and glucose transporters, or hormones, such as leptin and gherlin also take part, as they are associated with food intake.

Cervantes et al. [1] followed the reactions of pigs during 21-day HS. Body temperature, respiratory measurements, the expression of amino acid and glucose transporters, heat shock proteins, gherlin and leptin genes were studied. Lysine and glucose are two important transporter compounds because lysine is the primary limiting amino acid in pigs and glucose is an energy source. The gene expression of amino acid transporters (amino acid transporter -*bo* + *AT*, cationic amino acid transporter-*CAT1*) and glucose (Na-glucose transporter-*SGLT1*, glucose transporter-*GLUT4*) transporters may provide information on the possibility of basic metabolic and energy disruptions following HS. According to their results [1], the expression of *HSP90*, *CAT1*, *SGLT1* and *GLUT4* genes increases due to HS, and the voluntary food intake remains low despite the above-mentioned increase [1].

3. Applications of PFAs in Pig Production

Extracts from plants containing biologically active substances are of interest for their antiviral and antimicrobial effects. Laboratory tests have shown the antioxidant effect of oregano, thyme, clove, pepper, lavender, and basil [8,9,48,49]. Due to the phenolic hydroxyl group in thymol, carvacrol and other plant extracts, they act as hydrogen donors, allowing the neutralization of peroxyl radicals formed as the first step of lipid oxidation [50].

According to the categorization of Mendel et al. [51] the most important groups of phytogens are tannins, saponins and polyphenols. Polyphenols are the major bioactive compounds in most plant extracts, but their composition and concentration may vary greatly by plant species and in different plant parts, due to the geographical origin, harvest period, environmental and storage factors, and processing methods. In the most commonly used plant extracts, the main ingredients are anethole, capsaicin, carvacrol, cinnamon-aldehyde, curcumin, eugenol and thymol [52]. The literature data show that the addition of phytogenic substances (spices, herbs or extracts) as feed supplements may affect certain parameters related to the growth and health of swine, thereby improving the growth performance in pigs [53].

Growth performance is economically the most important trait [54] affected by PFAs. Czech et al. [54] used an herbal extract including garlic bulbs (*Allium sativum* L.), common licorice roots and tillers (*Glycyrrhiza glabra* L.), common thyme herb (*Thymus vulgaris* L.) and caraway fruits (*Carum cavi* L.), for fattening pigs. The control group was fed with standard mixtures (Starter, Grower, Finisher)

whereas group II and III received the same mixtures, but supplemented with the antibiotic avilamycin or the herbal extract (0.8 g kg^{-1} feed), respectively. According to their results, the average daily weight gains (798 g with herbal extract vs. 812 g with avilamycin and 748 g in the negative control group), feed conversion ratio (2.66 vs. 2.63 and 2.88, respectively) and blood parameters proved that this herbal preparation may serve as an alternative for antibiotics. Furthermore, Grela et al. [55] used inulin supplementation combined with garlic resulting significantly higher daily weight gains than in the control.

PFAs are possible to utilize in different growth periods of swine, such as the nursery, [47] growing, [54] and finishing period [55] in order to promote the healthy development during fattening. Liu et al. [47] supplemented a nursery basal diet of pigs with 10 mg/kg of capsicum oleoresin, garlic botanical, or turmeric oleoresin. Their results showed a differentiation in the regulation of the expression of genes in the *ileal mucosa* of pigs, enhancing the health of gut mucosa and stimulating the immune system [47]. In an experiment on a Hungarian pig farm in Transdanubia, the phytogenic additive improved the feed intake of sows and the mean weight of rearing pigs was significantly higher in the group that consumed plant extracts than in the control group [56]. Grela et al. [55] lowered the cholesterol in the blood and longissimus muscle, and increased the omega-3 and omega-6 fatty acids in the longissimus muscle of pigs using a supplement of a mixture of inulin and garlic. *Echinacea purpurea* supplementation [54] resulted in a significantly higher number of lymphocytes and total leucocytes in groups of fattening pigs receiving an ethanolic extract for five days, indicating the immune stimulating effect of *Echinacea*. Alfalfa (*Medicago sativa* L.) has positive health effects such as detoxification, and immune system stimulation thanks to the broad range of constituents, like different pigments, such as carotenes, xanthophylls and chlorophyll, and organic acids, such as fumaric acid or saponins [57]. Pietrzak and Grela [58] used an alfalfa protein concentrate (control, 1.5%, 3%, 3% periodically), and observed beneficial effects in the growing and finishing period of pigs in terms of their red blood cell indices: haematocrit, red blood cell count, and hemoglobin concentration, along with an increased number of white blood cells in the serum. Nevertheless, the enzymes activities of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase in the blood plasma indicated a negative effect on the liver.

Furthermore, alleviation of the effects of worms is also possible with the application of PFAs. As reported, the number of worms (*Ascaris suum*) was slightly reduced by a 47-herb-mixture in the intestinal tract, without affecting the performance of the pigs [59].

4. Effect of Phytogens on Pigs under Stress Conditions, with Special Regards to HS

As seen in the former section, PFAs may exert beneficial effects on pig metabolism and can contribute to the maintenance of their health, but doubt remains as to their effectiveness under stressful conditions. A possible way to prevent or attenuate adverse processes may be to use phytogenic additives [60].

According to the review works of Franz et al. [61] and Mohsen and Kim [62], the reasons for using PFAs in pigs are that they have growth-promoting effects and a beneficial influence on palatability and gut function. The effects of PFAs mostly benefit the gut through eliminating some pathogen germs, which is crucial in stress situations. PFAs stabilize and enhance the microbial constituents, making the intestinal system more resilient towards undesired events [63].

Besides the aforementioned, van der Klis and Vinyeta [64] extended this summary with improved nutrient utilization, benefits on intestinal mucosa, increased enzymatic activity in the intestinal tract and positive effects on reproductive performance as well.

Furthermore, PFAs can be effectively used for overcoming the negative effects of certain stressors, such as transport [34] or HS [65], or of pathogens [58]. Zhang et al. [34] investigated the effects of HS on the mRNA expression of HSPs (*HSP27*, *HSP70*, *HSP90*) with stress-free and transport stressed pigs. Draskovic et al. [57] found that a mixture of an essential oil blend consisting of *Thymus vulgaris*, *Origanum vulgare* and *Coriandrum* sp. essential oils and an extract of *Castanea sativa*, combined with

lysozyme and nicotinamide as immunomodulators, was also effective against *Lawsonia intracellularis* infection [66].

Liu, Tong and Zhou [46] reviewed the effects of Chinese herbal feed additives on animal production and summarized positive effects in antimicrobial actions, enhancing immune functions, anti-oxidative action, and a beneficial effect on meat flavor and gut functions [46]. Song et al. [67] also used traditional Chinese medicine decoction made up of four components: *Cortex Phellodendron* (Huangbai), *Rhizome Atractylodes* (Cangzhu), *Agastache rugosa* (Huoxiang) and *Gypsum fibrosum* (Shigao) in a dry weight ratio of 1:1:1:0.5. Glucose uptake and digestion in the small intestine improved after six days of treatment, while the blood glucose level remained unchanged. Dong et al. [46] examined the changes induced in hormonal and gene expressional level: after six days of HS, the dietary supplementation using the same Chinese medicinal prescriptions as Song et al. [67] improved porcine growth performance significantly and decreased the level of the stress hormone cortisol. As for gene expression, the medicinal treatment resulted in the up-regulation of genes neuropeptide Y, motilin and secretin and the down-regulation of glucagon expression in the pig jejunum [46].

Frankič et al. [68] have shown that supplementing fattening pigs with plant extracts has reduced the DNA degradation of lymphocytes, indicating a mitigating effect of oxidative stress with phytopharmaceuticals. Zhang et al. [34,48] treated pigs exposed to transport stress, which is often coupled with HS, with oregano extract (main active ingredients: carvacrol and thymol) and found that the total antioxidant capacity and superoxide dismutase activity increased in the oregano supplemented animals. Cheng et al. [69] also used an oregano extract in their experiments, which increased the activity of the antioxidative enzymes in the muscles, suggesting increased oxidative stability. In addition, intramuscular fat content increased and the proportion of fatty acids shifted towards polyunsaturated fatty acids (PUFAs) [69].

Lan and Kim [22] evaluated the effect of dietary essential oils (EO) and betaine on growth performance, nutrient digestibility and serum hormones in growing-finishing pigs under cyclical HS conditions: (control: 23 °C for 24 h; cyclical HS: 37 °C from 10:00 to 19:00 h and 23 °C from 19:00 to 10:00 h). They concluded that dietary EO may be effective in the alleviation of HS in growing-finishing pigs, since at week 12 and 18, dietary EO decreased serum cortisol and norepinephrine concentration, and EO and betaine decreased epinephrine concentration [22].

5. Determination of Sample Type and Sampling Frequency in HS Investigations in Pigs

According to the reviewed literature, methods related to the investigation of the antioxidative system, feed intake and transport process are sufficiently broad for the proper characterization of HS-induced changes, as well as for the investigation of the possible easing effect of PUFAs. However, the experimental set up, methodology and sampling frequency should be considered thoroughly both from an experimental and an animal-welfare point of view, since most sampling processes are invasive techniques. In cases where the purpose is to characterize the whole growth period, sampling only at the final stage—when pigs are raised to slaughter weight—is probably not sufficient. All this raises questions about the necessity of tissue sampling after slaughter and/or by biopsy.

Sampling Conditions and Experimental Results for the Investigation of Heat Stress

Xin et al. [45], tested a number of 27 80-day-old pigs subjected to neutral and elevated temperature with ad libitum feed intake, or limited feed intake in case of the heat-stressed pigs exposed to heat shock. Blood was collected from pigs on days 3, 7, 14, and 21 of the experiment. Parameters related to the analysis of the antioxidant system (changes in enzyme activity, total antioxidant capacity, lipid peroxidation) were mostly determined from blood serum, and in some cases from liver tissue. In the case of blood sampling, there was an experiment during which blood was collected not only at the time of slaughter but also at four intermediate times [45].

Cervantes et al. [1] kept 70-day old pigs under HS conditions for 21 days, then 2.5 h after the last meal, all pigs were slaughtered, and samples were taken from the liver, muscle tissue, and adipose

tissue (from the subcutaneous white adipose tissue around the 10th and 11th ribs), as well as a mucosal sample from the jejunum and the duodenum for expression studies of *HSP70*, *LEP* and *GHRL* genes.

Zhang et al. [48] submitted fattening pigs weighing 50 ± 2 kg (about four months of age) of *Erhualian* × *Pietrain* genotype to transport stress of different duration (0, 1, 2 or 4 h), and then the animals were slaughtered. The aim of the study was to measure the change of mRNA expression of the *HSP70* and *HSF-1* genes, as well as to measure the activity changes in enzymes indicating muscle damage. After slaughter, blood was taken and stored at -20°C before serum enzyme activity measurements (creatine kinase (CK) and aspartate aminotransferase (AST)). In addition, sampling of the heart, liver and abdomen (0.5 g, -70°C) was performed. According to their results, the activity of the AST and CK enzymes increased significantly after 1 and 2 h post transport, and the expression of *HSP70* was different in different tissues: in the heart and liver, it did not change significantly, while an increase occurred in the abdomen.

Zhang et al. [34] investigated the effects of oregano oil (OO) following the transport of pigs. The experiment started with 120-day animals. The aim is to investigate whether OO had the effect of relieving stress and increasing the antioxidant capacity. The 180 animals were divided as follows: 200 mg kg^{-1} vitamin E (VE) or 25 mg kg^{-1} OO. Each group was divided into two subgroups: stress-free (NS) or transport-stressed (TS). At the end of the 28-day period, 72 pigs were slaughtered after electrical stunning, and blood and liver samples were taken immediately. The transport affected the metabolism and the functioning of the immune system. Serum cortisol and norepinephrine concentrations in pigs exposed to transport stress were significantly reduced in the OO diet. The serum and liver showed significantly increased reactive oxygen and MDH levels, indicating lipid peroxidation. Serum glutathione peroxidase (GSH-Px) was significantly increased in the dietary treatment. Liver SOD increased dramatically, regardless of the effect of transport or dietary treatment (Table 2). Liver heat shock protein *HSP27* and *HSP90* increased significantly after delivery. These results suggested that oregano essential oil is beneficial in terms of alleviating transport stress and improving antioxidant activity, similar to VE. Cottrell et al. [70] summarized the beneficial health effects of cinnamon, especially in the treatment of diabetes. Pigs were fed a diet of 12.5 g/kg cinnamon during their finishing phase and subjected to intravenous glucose tolerance tests; after slaughter, gene expression studies were performed on the muscle tissues. According to their results, cinnamon supplementation increased daily weight gain (0.93 vs. 1.03 kg/d) and improved the feed conversion ratio (2.69 vs. 2.48). As for the glucose clearance, it was increased by dietary cinnamon (103 vs. 74 mmol·min/L), which indicates a probably beneficial role in alleviating HS [45].

Chen et al. [71], kept 12 six-month-old male Bama miniature pigs for eight days at 25 or 40°C . After 8 days of heat treatment, the pigs were slaughtered. Hepatic immunohistochemical and antioxidant enzyme activity, and MDA and H_2O_2 levels were measured and an RT-PCR analysis of NAD(P)H quinone-oxidoreductase 1, SOD1; heme oxygenase-1-HO-1, and the glutamate cysteine ligase catalytic subunit (GCLC) was conducted. The activity of superoxide dismutase, glutathione peroxidase, catalase enzymes, and glutathione content increased as a result of heat, as well as the expression of NRF2-regulated genes, suggesting a high heat tolerance of the liver of the Bama miniatures.

Wu et al. [72] took blood samples (10 mL) from the jugular vein of 28-day-old piglets, 1 h after the last feed intake. Immediately after blood collection, the pigs were anesthetized and bled with intravenous sodium pentobarbital (50 mg/kg body weight). For gene expression studies, samples of the *ileal mucosa* were used to examine expression changes of *HSP70* gene (Table 2), and blood plasma was used to determine the amino acid concentration (Table 1).

Table 2. Sampling conditions for the investigation of HS-related gene expression in pigs.

| Frequency of Sampling | Weight of Animals (kg) | Conditions of Slaughter | Investigated Gene Expression | Sample Type | Age of Animals/Duration of Experiment (Days) | Ref. |
|-----------------------------|------------------------|----------------------------------|--|--|--|------|
| at slaughter | 32.6 ± 3.2 | bleeding after electric stunning | <i>HSP70, GHRL LEP</i> | duodenum, liver, muscle intestinal mucosa, adipose tissue | 70/21 | [1] |
| at slaughter | 50 ± 2 | bleeding after electric stunning | <i>HSP27, HSP70, HSP90</i> | liver | 120/28 | [34] |
| at slaughter | 50 ± 2 | 3% sodium pentobarbital | <i>HSP70</i> | heart liver, stomach | 120/0 | [44] |
| | | | | Nrf2=nuclear factor erythroid 2-related factor 2; NQO1=NAD(P)H: quinone-oxidoreductase 1; SOD1=superoxide dismutase 1; HO-1=heme oxygenase-1; GCLC=glutamate cysteine ligase catalytic subunit | | |
| at slaughter | 10.79 ± 0.03 | data not available | IGF-1 receptor, IGFBP-1, IGFBP-3 genes | liver | 180/8 | [70] |
| on the 22nd day | 40.8 ± 2.7 | bleeding after electric stunning | | liver | 80/22 | [68] |
| at slaughter | 79 ± 1.5 | bleeding after electric stunning | carnosine synthetase (<i>CARNS1</i>) | <i>longissimus dorsi</i> muscle | data not available /21 | [41] |
| 1st, 7th days of experiment | 28 ± 3 | data not available | <i>HSP70, GIF-1α, GPx-1, GPx4</i> | leucocyte | data not available/7 | [36] |
| at slaughter | 63.8 ± 2.9 | pentobarbital injection | <i>SOD1, SOD2, CAT</i> | red portion of the semitendinosus muscle | data not available 2, 4, 6 h | [43] |
| at slaughter | 24.64 ± 3 | pentobarbital injection | <i>HSP27, HSP70, HSP90, pyruvate dehydrogenase kinase (PDK4)</i> | small intestine | data not available /12 h | [5] |
| at slaughter | 114 ± 2 | data not available | <i>CuZnSOD</i> | <i>longissimus dorsi</i> muscle, backfat, liver | data not available /6, 9, 12 months | [30] |
| at slaughter | 7.10 ± 0.52 | head-only electric stun tong | <i>HSP70, NPY (neuropeptide Y), MLN (motilin) GCG (glucagon) NPY, MLN, SCT (secretin) SST (somatostatin)</i> | jejunum | 60/1, 6 | [47] |
| at slaughter | 7.15 ± 0.58 | anesthesia | <i>SGLT1, GLUT2</i> | duodenum and jejunum | 60/10 | [65] |
| at slaughter | 77.80 ± 4.25 | electrical stunning | <i>HSP27, HSP70, HSP90</i> | liver | data not available/28 | [33] |

The literature revealed that HS and the effect of PFAs was mostly investigated through some elements of the antioxidative system [32,36,41–43] but other metabolites were also investigated, such as hormones [15], or compounds related to amino acid [58] or glucose [47] metabolism. As for related gene expression, mostly *HSPs*, and antioxidant enzyme genes were examined in several cases [1,5,34,43,44], where positive HS-alleviating effects of PFAs were found [59–67].

Based on the reviewed literature, sampling for investigation of metabolites, the antioxidative system (Table 1) and HS-related gene expression (Table 2) involves quite a range of experimental conditions in terms of sampling frequency, the weight of animals, time and conditions of slaughter, sample tissue type and the investigated parameters. It became obvious that sampling mainly happened at the time of slaughter. Probably, this is an experimental practice due to animal welfare considerations. Blood and fecal sampling were two exceptions—these sampling techniques were applied during the

experiment because they are easy and harmless to collect, and it is important during a stress-related experiment to remove other sources of potential stress agents [15,45,46]. In the molecular biology studies, only tissue samples (liver, muscle, fat and heart tissue, intestines and abdomen) were taken in these experiments for gene expression assays. Biopsy, as a sampling opportunity, was not used in any of the experiments in the reviewed literature that left out the possibility of tissue measurements during the experiment, only at the endpoint. This drawback was overcome by the slaughter of animals at certain points during the experiment.

The reviewed investigations show that although the age of the animals, the duration of HS, and the investigational methodology varied widely, sampling did not generally occur during the experiment, only at the end. This raises the question of how to approach HS-related consequences in a more unified way, since, during an experiment, animals are subjected to HS for a period of time: investigation at the end point is clearly not enough. Tissue biopsy would be ideal for elucidating HS-related processes during the different developmental stages of pigs, besides the data acquired at the time of slaughter. Almost all of the parameters listed in this review would be possible to measure using tissue samples: antioxidant capacity, antioxidant enzyme activities, gene expression studies of heat shock proteins, and genes related to metabolism. If avoiding any invasive techniques, urine samples would also be suitable for the periodical detection of antioxidant capacities in the animals.

It would be advisable to measure antioxidant capacities along with lipid oxidation, as these methods are easy and require only a UV/VIS spectrophotometer. The determination of SOD, CAT and GPx would definitely provide a good overview of the antioxidant enzyme system. Furthermore, as the stressor is heat, the measurement of the expression of HSPs is unavoidable on a protein and gene level as well. Finally, if the possibilities are used, the gene expressions underlying the antioxidative processes mentioned above may also be beneficial to determine.

These approaches, taken together, would contribute to a more complex understanding of the effect of PFAs in alleviating HS.

6. Conclusions

On the basis of the literature review, it can be stated that in the last few years, special attention has been paid to PFAs in animal feeding, especially to compensate for transport and heat stress, which have a negative effect on meat quality as well as on the efficiency of production. The use of PFAs is based on their high antioxidant content, which can be beneficial in alleviating various stress conditions, so as to protect against increasingly frequent HS due to climate change. There are literature references for the beneficial effects of PFAs, but their mode of action requires extensive further analysis, also their effect was tested against HS in only a few cases. These facts justify the intention of elucidating the alleviation mechanisms of PFAs against HS.

As for sampling frequency, it would be useful to include tissue samplings for gene expression studies along with blood sampling. This approach would not only help with understanding the outcome of the experimental period, but also would give us insight into the dynamics of stress evolution as well as the alleviating role of phytogens during this period. All these variables play a role in determining the stress-reducing effects and dynamics of phytogens, consequently contributing to an understanding of the mode of action of PFAs and providing useful information on possible feed development trends.

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