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Running title: Plant compounds for piglets to face weaning

**Effect of the supplementation with a combination of plant extracts on sow and piglet performance and physiology during lactation and around weaning<sup>1</sup>**

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## **Lay summary**

Weaning is a critical period for piglets. Some plant extracts, known to exhibit antioxidant, anti-inflammatory or anti-bacterial properties, may improve piglets' robustness at weaning. This study investigated the effects of a maternal and/or a direct supplementation of piglets with a combination of plant extracts on sow and piglet metabolic, immune, inflammatory and oxidative status during lactation and around weaning. The maternal supplementation corresponded to a powdered supplement top-dressed on sow diet during late gestation and lactation and a liquid solution administered once one week before parturition. The piglet supplementation was a liquid solution administered once on day 3 of age. The most concentrated components of the powder were extracts of fenugreek, siberian ginseng and cat's claw. The liquid solutions contained mostly oregano and eucalyptus essential oils. The maternal supplementation had few effects on sow immune, inflammatory and oxidative status but modified milk composition at the end of lactation. It did not improve growth performance and the immune, inflammatory and oxidative blood parameters of piglets around weaning. The direct supplementation of piglets modified their immune and oxidative status before weaning and increased their growth performance during the post-weaning period, showing the potential of plant extracts as part of preventive strategies dedicated to improve piglets' robustness during the suckling and post-weaning periods.

## **Teaser text**

Supplementing piglets with a combination of plant extracts improved their immune and oxidative status before weaning and their growth performance during the post-weaning period, showing the potential of plant extracts to improve piglets' robustness during the suckling and post-weaning periods.

## **Abstract**

Weaning is a critical period for pigs. Some plant extracts showing antioxidant, anti-inflammatory or anti-bacterial properties, provided to piglets and/or their dam, may improve piglets' robustness at weaning, thus reducing the need for antibiotics. This study investigated the effects of a maternal and/or a direct supplementation of piglets with a combination of plant extracts on sow and piglet performance and their metabolic, immune, inflammatory and oxidative status during lactation and around weaning. Sixty-four sows were assigned to the control or treated group. Treated sows were supplemented with a powdered plant extracts supplement daily top-dressed on feed from day of gestation (**DG**) 106 to day of lactation (**DL**) 28 and a liquid solution top-dressed on feed on DG109. Within each sow group, litters were divided into two groups: a control piglet group and a treated piglet group. A single dose of a liquid solution was orally given to piglets in the treated piglet group. Piglets were weaned on DL28. Blood samples were collected from sows on DG94, DG112 and DL26 and from 2 piglets per litter on DL3, DL14, DL25 and 5 d post-weaning to analyze indicators of metabolic, immune, inflammatory and oxidative status. Colostrum and milk samples were collected at farrowing, DL6 and 26. Maternal supplementation had no effect on sow metabolic, immune, inflammatory and oxidative status except for fewer lymphocytes on DG112 ( $P < 0.05$ ) and a lower plasma concentration of non-esterified fatty acids on DL26 ( $P < 0.05$ ). Maternal supplementation tended to decrease dry matter and gross energy ( $P < 0.10$ ) and reduced fat and haptoglobin concentrations ( $P < 0.01$ ) in milk on DL26. Maternal supplementation had no effect on piglets' growth performance and blood indicators during lactation and around weaning. On DL25, the direct supplementation of piglets decreased their neutrophils proportion ( $P < 0.05$ ), increased the expression of genes encoding pro- and anti-inflammatory cytokines in whole blood culture in response to LPS ( $P < 0.05$ ) and tended to decrease the oxidative stress index

( $P = 0.06$ ). After weaning, these beneficial effects were no longer observed but the supplementation improved piglets' growth performance during the post-weaning period ( $P < 0.05$ ). Plant extract supplementation could thus modify the composition of mammary secretions and improve post-weaning performance of piglets potentially related to the modification of their immune and oxidative status before weaning.

**Key words:** colostrum, essential oil, growth performance, milk, plant extract, weaning

**Abbreviations:** ADG, average daily gain; BAP, biological antioxidant potential; BW, body weight; DG, day of gestation; DL, day of lactation; DPW, day post-weaning; dROM, reactive oxygen metabolites; EDTA, ethylenediaminetetraacetate; IFN- $\alpha$ , interferon- $\alpha$ ; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NEFA, non-esterified fatty acids; ODN, CpG oligodeoxynucleotide; OSI, oxidative stress index; PI, proliferative index; TLR, toll-like receptor.

## **Introduction**

Early weaning is a critical period for piglets that is characterized by transiently altered growth performance and a high incidence of post-weaning diarrhea (Pluske et al., 1997). Over the past decades, antibiotics have been widely used in the swine industry worldwide to prevent post-weaning diarrhea, participating in the spread of antimicrobial-resistant bacteria (Lekagul et al., 2019). The growing number of these antimicrobial-resistant bacteria in both farm animals and humans have raised public health concerns and highlighted the emergency to rationalize the use of antibiotics (i.e., use antibiotics only to treat, not prevent the development of bacterial diseases). To support the reduction of antibiotic utilization in swine industry, and most notably in weaned pigs, feeding strategies have been implemented to maintain piglet health and performance at weaning.

In this context, plant extracts, used as feed additives in sow and piglet diets, have gained interest as part of preventive strategies dedicated to improve the ability of piglets to face the weaning challenge. Indeed, some plant extracts may exert antimicrobial (such as oregano, thyme and eucalyptus extracts), antioxidative (such as oregano, rosemary and sage extracts), immune system-promoting and anti-inflammatory properties (such as lavender, eucalyptus and rosemary extracts), and thereby improve pig health and performance at weaning (Mroz, 2005; Zeng et al., 2015; Omonijo et al., 2018). For this purpose, synergistic combination of plant extracts are used in dam or piglet supplementation. The supplementation of sow feed with plant extracts during gestation and/or lactation has been shown to improve health and performance of the offsprings during the suckling period (Tan et al., 2015; Balasubramanian et al., 2016; Parraguez et al., 2021). The beneficial effect of maternal supplementation could be due to the improvement of the physiological status of the sows (Wang et al., 2019; Reyes-Camacho et al., 2020) and the nutritional, immune or antioxidant quality of colostrum and milk (Lee et al., 2013; Meng et al., 2018; Wang et al., 2019). Moreover, bioactive compounds of plant extracts

can be transferred into colostrum and milk of supplemented sows (Val-Laillet et al., 2018; Reyes-Camacho et al., 2020) and could then have a direct effect on the piglets. In weaned piglets too, adding plant extracts to the diet had positive effects on their growth performance and health resulting from the improvement of their immune, inflammatory and oxidative status (Su et al., 2018; Xu et al., 2020).

In the present study, we investigated the potential benefit of a preventive strategy based on plant extracts selected for their potential health-promoting properties and provided to either the sow, the piglet or both, to improve piglets' robustness at weaning. We hypothesized that these supplementations would improve the physiological status of piglets during the suckling and post-weaning periods, and in turn their post-weaning growth performance, through direct positive effects (piglet supplementation) or by improving the physiological status of their dam and the nutritional and immune composition of the colostrum and milk (maternal supplementation). We also investigated whether maternal and piglet supplementations could have positive synergistic effects.

## **Materials and methods**

The experiment was conducted in the INRAE experimental facilities « Physiologie et Phénotypage des Porcs » (INRAE, 3P, 35590 Saint-Gilles, France, <https://doi.org/10.15454/1.5573932732039927E12>) in compliance with the ARRIVE animal experimentation guidelines and the European regulation on animal experimentation (2010/63). The experiment was evaluated by the regional Ethics Committee in Animal Experiment of Rennes and got the authorization from the French Ministry of Higher Education, Research and Innovation (authorization APAFIS#22515-2019101909586795 v4).

## ***Animals and experimental design***

Sixty-four Landrace x Large White sows (from parity 1 to 7) and their litter were included in this study in 4 batches of 15 to 17 sows, with a batch corresponded to a group of sows inseminated on the same days. Because of the lockdown imposed by the French government as part of the management of the COVID-19 crisis, the biological samples of the second batch of the experiment were not collected after the day of lactation (**DL**) 14. Sows were inseminated with semen from Pietrain boars. On day of gestation (**DG**)  $93 \pm 1$  (DG0 being the day of the first insemination), sows were assigned to either the sow-control (**sCTRL**, n = 32) or the sow-treated group (**sTRT**, n = 32) based on parity, body weight (**BW**) and backfat thickness. From the day of transfer from gestation to farrowing pens (DG106) to the end of lactation on DL28 (DL0 being the day of parturition), sTRT sows were supplemented with 25 g/d of a powdered supplement and received once 20 mL of a liquid solution for sows on DG109. The powdered supplement contained plant extracts (62.5 g/kg of fenugreek (*Trigonella graecum*) seed extract, 50.0 g/kg of siberian ginseng (*Eleutherococcus senticosus*) root extract, 41.7 g/kg of cat's claw (*Uncaria tomentosa*) root extract, 8 g/kg of artichoke (*Cynara scolymus*) leaf extract, 11.6 g/kg of rosemary (*Rosmarinus officinalis*) extract, and 2.5 g/kg of milk thistle (*Silybum marianum*) seed extract), 0.85 g/kg of vitamin C and 0.85 g/kg of vitamin E, and 82.5 g/kg of magnesium chloride. It was daily top-dressed on the diet of sTRT sows during the morning meal (0800h). The liquid solution for sows contained essential oils of eucalyptus (*Eucalyptus globulus*, 18.75 mL/L) and oregano (*Origanum vulgare*, 7.5 mL/L), and vitamin C (15 mL/L). It was top-dressed on the afternoon meal (1330h) on DG109. Sows in the sCTRL group were not supplemented. Within each sow group, half of the litters were assigned to the piglet-control group (pCTRL) and the other half to the piglet-treated group (pTRT). All the piglets of the pTRT litters received 2 mL of a liquid solution for piglets 3 days after birth whereas the pCTRL litters did not receive this solution. The litters were thus allocated in four



experimental groups: sCTRL-pCTRL, sCTRL-pTRT, sTRT-pCTRL, and sTRT-pTRT. The liquid solution for piglets contained essential oils of eucalyptus (*Eucalyptus globulus*, 12.5 mL/L) and oregano (*Origanum vulgare*, 5 mL/L), and organic acids (30 mL/L of citric acid, 30 mL/L of caprylic acid and 0.4 mL/L of ascorbic acid). It was orally administered to the piglets with a serynge. The powdered supplement and liquid solutions for sows and piglets were supplied by IDENA (Sautron, France). Their components were selected for their potential or demonstrated immune system-promoting, anti-inflammatory, antioxidative, antibacterial and anticoccidial properties.

### ***Sows and litters management***

Housing and management practices from the insemination to weaning were similar for sows in both treatments. Sows were transferred to two farrowing rooms on DG106. Sows were kept in individual crates with slatted floor (2 by 2.5 m) and equipped with two infrared heat bulbs. The lactation rooms were environmentally controlled to keep the ambient temperature between 24 and 25 °C. During gestation and until the day of farrowing, sows were fed a standard gestation diet (providing as fed-basis 9.7 MJ of net energy/kg, 13 % crude protein, 0.6 % lysine and 5 % crude fiber) through an automatic feeder in free access. Feed allocation was between 2.4 and 3.3 kg daily, depending on sow parity, body condition and backfat thickness. From DL1 until weaning (on DL28), sows were fed a standard lactation diet (providing as fed-basis 9.5 MJ of net energy/kg, 16.2 % crude protein, 1.0 % lysine and 4.4 % crude fiber) distributed in 6 meals daily through an automatic feeder. They received between 2.8 and 3.2 kg on DL1 and then feed allowance was increased by 1 kg/d until ad libitum feeding, which was reached approximately on DL4 or DL5. From DG106 and throughout lactation, feed refusals were weighed daily and actual feed intakes were calculated. Water was available ad libitum throughout the experiment.

Farrowing was induced on DG115 by an i.m. injection of prostaglandin F2 $\alpha$  (2 mL of Dinolytic, Zoetis, France). If the sow farrowed prior or was visibly close to farrowing, this procedure was omitted. Within 24 h after birth, each piglet was identified by an ear clip, tail docked and received an i.m. injection of iron. Cross fostering, if needed, was performed intra-experimental group within 2 d after birth. During lactation, piglets had free access to water and had access to prestarter feed from 21 d of lactation.

Piglets (189 sCTRL-pCTRL, 186 sCTRL-pTRT, 192 sTRT-pCTRL and 182 sTRT-pTRT) were weaned at  $28 \pm 1$  d of age (DL28), vaccinated against porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* (Porcilis<sup>®</sup> PCV M Hyo, MSD Santé Animale, France), and transferred into a post-weaning unit where they were housed in collective pens of 9 to 12 piglets with slatted floor (2.7 x 1.5 m). The pens were located in rooms of 6 and 12 pens with the same number of pens per experimental treatment in each room. Each pen housed only pigs from the same experimental treatment (sCTRL-pCTRL, sCTRL-pTRT, sTRT-pCTRL or sTRT-pTRT). Since robustness is revealed in challenging environments (Friggens et al., 2017) and because weaning conditions may be more challenging in commercial farms than in experimental units, piglets were weaned in health-challenging conditions. These challenging weaning conditions were expected to reveal the potential beneficial effects of the maternal and piglet supplementations on piglet robustness at weaning. For that purpose, piglets with a similar range of BW from at least four litters of the same experimental treatment were mixed in the same pen to induce a social stress. Piglets were assigned to a pen using the weaning BW as the main factor, and litter as the second factor. Moreover, piglets were transferred in post-weaning rooms that were not disinfected nor cleaned after occupation by piglets from the previous batch. Finally, the ambient temperature of the post-weaning rooms was transiently non-optimal (i.e., set at 24 °C when the piglets arrived and then progressively increased to 28 °C in 4 to 6 h). The piglets stayed in the post-weaning facilities until day post-weaning (**DPW**) 35 (DPW1 being

the day of weaning) that corresponded to the end of the experiment. Each pen were equipped with troughs and cup drinkers. Piglets were offered the prestarter feed for the first 5 d and then the starter diet until DPW35, with a 3-d transition period between the two diets. The postweaning diets were not supplemented with antibiotics. Feed and water were available ad libitum during this period.

### ***Measurements and samplings on animals***

***Measurements.*** Sow BW and backfat thickness were recorded on DG93, DG106, after parturition (DL0) and on the day of weaning (DL28). Backfat thickness was measured ultrasonically at the P2 site of the sow on both left and right flanks 6.5 mm away from the spine. All piglets were counted, weighed, and sexed within 24 h after birth. Piglets were also counted and weighed on DL6, at weaning (DL28) and at the end of the post-weaning period (DPW35) to calculate their average daily gain (**ADG**). Regarding mortality, the date and piglet weight at death were also recorded.

***Blood sampling.*** Blood samples were collected from the jugular vein of all sows after an overnight fasting on DG94 (i.e., before treatment), DG112 (i.e., 6 d after the beginning of the powdered supplement distribution and 3 d after the liquid solution administration to sows), and DL26. Tubes coated with sodium heparin (BD Vacutainer Systems, Plymouth, UK), were used to collect samples (9 mL) for the measurement of plasma concentrations of metabolites (non-esterified fatty acids (**NEFA**), glucose, lactate, urea, and creatinine), and of indicators of the oxidative status (reactive oxygen metabolites, **dROM**, and biological antioxidant potential, **BAP**). Tubes coated with ethylenediaminetetraacetate (**EDTA**, BD Vacutainer Systems) were used to collect samples (9 mL) for white blood cell count determination and the measurement

of the inflammatory status indicator haptoglobin. An additional blood sample (9 mL) was collected in a heparinized tube (BD Vacutainer Systems) on DL26 for the lymphocyte proliferation test and the measurement of gene expression of cytokines in whole blood cell cultures.

Within each litter, two pairs of non-adopted piglets (one female and one male in each pair) were selected on a live-weight basis to have birth weights closest to the average birth weight of the litter. Blood samples (4 mL) were collected from the jugular vein of piglets of the first pair twice during the perinatal period: on DL3 (i.e., before the administration of the liquid solution for piglets) and on DL14, with a tube coated with sodium heparin for the measurements of immunoglobulins (Ig) G and M, and interferon- $\alpha$  (IFN- $\alpha$ ), three indicators of the immune status of piglets. The concentration of IgM was measured only on DL14. The second pair of piglets was blood sampled twice during the peri-weaning period: 3 d before weaning on DL25 and 5 days after weaning on DPW5. Tubes coated with sodium heparin (4 mL) were used for the measurements of IgG, IgM, BAP and dROM and tubes coated with EDTA (4 mL) for the measurement of haptoglobin. An additional blood sample (9 mL) was collected from the female of the second pair of piglets on DL25 and DPW5 with tubes coated with sodium heparin for the lymphocyte proliferation test and the measurement of gene expression of cytokines in whole blood cell cultures.

Heparinized blood samples for the lymphocyte proliferation and cytokine expression assays were kept at ambient temperature and used shortly after being collected. The other blood samples were kept on ice. A 100- $\mu$ L fraction of blood sample from EDTA tubes was used for white blood cell count determination. Plasma was then separated by centrifugation at 2,500 x g for 10 min at 4 °C and stored at -20 °C until analyses.

For blood samplings, sows were restrained with a snout rope and piglets were maintained on the back (manually on DL3 and DL14, and through a V-shaped restrainer on

DL25 and DPW5). The restraining period was limited to 2 min to limit excessive stress and pain. If collection was unsuccessful after such time, it was stopped, and sample was deemed as a lost blood sample.

***Colostrum and milk sampling.*** A colostrum sample (70 mL) was collected within 2 h after the birth of the first piglet and milk samples were collected on DL6 and DL26. For milk collection, piglets were isolated from the sow for 45 min before collection. On DL6, milk samples (90 mL) were collected after an intramuscular injection of 20 IU of oxytocin (Biocytocin, Biové Laboratoires, Saint-Omer, France). On DL26, 10 IU of oxytocin (Biové Laboratoires) was intravenously injected in the ear of sows and sows were milked until milk ejection stopped. Colostrum and milk samples were manually collected from all functional teats, immediately filtered through a gauze and stored at -20 °C until composition analysis.

### ***Biological analyses***

***Plasma metabolites.*** Plasma concentrations of glucose, NEFA, lactate, urea and creatinine were determined using an automated multiparameter analyzer (Konelab 20i, ThermoFisher Scientific, Waltham, MA, USA) and commercial kits (provided by ThermoFisher Scientific, Ref. 981304, 981818, and 981811 for glucose, urea and creatinine, Wako Diagnostics, Mountain View, CA, USA, Ref. 434-91795 for NEFA, and Horiba ABX SA, Kyoto, Japan, Ref. A11A01721 for lactate). The intra-assay CV were between 0.9 and 1.9 %.

***Plasma indicators of inflammatory and oxidative status.*** Haptoglobin, an acute phase protein used as an indicator of inflammatory status, was assayed using a commercial kit (Phase™

Haptoglobine assay kit, Ref. TP801, Tridelata Development Ltd, Maynooth, Ireland) adapted to an automated multiparameter analyzer (Kone Instrument). The concentration of dROM, generated by the peroxidation of lipids, proteins or nucleic acids, and the total blood antioxidant potential (BAP), resulting from the combined effects of many antioxidants such as ascorbic acid, proteins, alpha-tocopherol or bilirubin, were assayed on plasma using commercial kits (dROM-test and BAP-test, Ref. MC003 and MC437 respectively, Diacron, Grosseto, Italy) adapted to an automated multiparameter analyzer (Kone Instrument) as previously described (Buchet et al., 2017). The intra-assay CV was 1.4% for dROM and 1.2% for BAP. An oxidative stress index (**OSI**) was calculated as the ratio between dROM and BAP.

*Plasma indicators of immune status.* The white blood cell count (i.e., the total numbers of white blood cells, and numbers and relative percentages of lymphocytes and neutrophils) were measured with a haematology automatic cell counter calibrated for pigs (MS-9<sup>®</sup>, Melet Schoesing Laboratoires, Osny, France). Plasma concentrations of IFN- $\alpha$  was assayed using the sandwich ELISA as described by Jamin et al. (2006). Plasma IgG and IgM concentrations were also determined by sandwich ELISA using goat anti-pig IgG or IgM (Biorad, Hercules, CA, USA, Ref. AAI41 for IgG and MyBioSource, San Diego, CA, USA, Ref. MBS224876 for IgM) diluted at 10 mg/L as capture antibody, horseradish peroxidase (**HRP**)-labeled goat anti-pig IgG or IgM (Biorad, Ref. AAI41P for IgG and MyBioSource, Ref. MBS 224946 for IgG) diluted 1:100,000 as detecting antibody, and native porcine IgG and IgM (ranging from 500 to 7.81 ng/mL, MyBioSource, Ref. MBS717028 for IgG and ranging from 1,000 to 15.62 ng/mL, Ref. MBS 238048 for IgM) as standards. Plasma samples were diluted at 1:400,000 (samples collected on DL3), 1:100,000 or 1:200,000 (samples collected on DL14) or 1:100,000 (samples collected on DL25 and DPW5) for IgG analysis and at 1:2,000 for IgM analysis in dilution

buffer (TBS with 0.05% Tween 20 and 1% BSA). The intraassay CV was 3.2% and 4.0%, and the interassay CV was 5.2% and 3.7% for IgG and IgM, respectively.

***Blood immune cell proliferation test and gene expression.*** The capacity of lymphocytes to proliferate after a mitogen stimulation was measured in blood samples collected from sows on DL26 and from piglets on DL25 and DPW5. First, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples on Histopaque 1077 and remaining erythrocytes were lysed with lysis buffer (sodium buffer containing 0.4% EDTA, 0.8% NH<sub>4</sub>Cl and 0.1% KHCO<sub>3</sub>, pH 7.4) and counted with an analyzer (Vi-Cell™ XR, Beckman Coulter, Paris, France). Mononuclear cells ( $2.0 \times 10^5$  cells/well) were cultured into 96-well flat bottomed cell culture plate, in the absence of mitogen or in the presence of concanavalin A (ConA) at 5 µg/mL to stimulate lymphocyte proliferation. Each culture condition was tested in quadruplicates, in a final volume of 200 µL. After 40 h of incubation in a 5% CO<sub>2</sub> humidified incubator at 37 °C, cultures were incubated with BrdU (10 µM/well) for 24 h. Then, BrdU incorporation was measured by ELISA using the cell proliferation ELISA BrdU colorimetric kit (Ref. 11669915001), according to the manufacturer's instructions. Briefly, plates were washed with PBS-0.5% Tween 20 and centrifuged 500 x g, at 18 °C for 10 min and then dried in an 5% CO<sub>2</sub> humidified incubator at 60 °C for 1 h. Cells were fixed by adding 200 µL of FixDenat in each well. After incubation at ambient temperature for 30 min, 100 µL of anti-BrdU solution was added in each well and plates were incubated at ambient temperature for 90 min. Plates were then washed thrice with washing buffer. Finally, 100 µL of tetramethylbenzidine substrate (TMB) was added for 10 min at ambient temperature and the reaction was blocked with 25 µL of H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 450 nm using a plate reader (Varioskan™ LUX, ThermoFisher Scientific). Media, mitogens and ELISA BrdU colorimetric kit were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Mitogenic

responsiveness of lymphocytes was expressed as a proliferation index ( $PI = (DO \text{ of stimulated cells} - DO \text{ of RPMIc}) / (DO \text{ of unstimulated cells} - DO \text{ of RPMIc})$ ).

We also investigated mRNA expression of toll-like receptors (TLR)-2, -4, and -9, and of 4 cytokines (i.e. interferon- $\alpha$  (IFN- $\alpha$ ), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , and -10) in whole blood cultures from blood samples collected on DL26 in sows and on DL25 and DPW5 in piglets. Whole blood was incubated in unstimulated conditions (medium alone), and after activation with agonists stimulating TLR-2 (Lipoteichoic acid from *Staphylococcus aureus*, LTA, Sigma Aldrich), TLR-4 (O55:B5 lipopolysaccharide from *Escherichia coli*, LPS, Sigma Aldrich) or TLR-9 (type A CpG oligodeoxynucleotide, ODN, Eurofins Genomics, Louisville, KY, USA). Briefly, heparinized total blood sample were diluted 1:2 in RPMIc and 400  $\mu$ L of diluted blood were cultured in triplicate into 24-well flat bottomed cell culture plate in the presence of 600  $\mu$ L of RPMIc, or treated with LPS, ODN or LTA (at 10  $\mu$ g/mL in the well). After 20 h of incubation in a 5% CO<sub>2</sub> humidified incubator at 37 °C, the 3 wells containing samples from the same animal and cultured in the same condition were pooled and rinsed with PBS. After a centrifugation at 700 x g at 4 °C for 10 min, 500  $\mu$ L of thiocyanate de guanidine DL lysis buffer (Macherey Nagel, Hoerdt, France) was added and samples were stored at -80 °C until RNA extraction.

After defrosting at room temperature, RNA was extracted and then purified using the column from the Nucleospin® 8 RNA blood kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. The amounts of total RNA extracted from blood samples were determined using a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Despite a concentration step performed with a speed-vac concentrator (ThermoFisher Scientific) for the low-concentrated samples, only samples reaching a concentration of 40 ng/ $\mu$ L were retained for real-time PCR analysis. The RNA quality was assessed with a Bioanalyzer (Agilent Technologies, Massy, France) using the RNA Integrity Number (RIN)



generated by Agilent 2100 Expert Software, version B.02 (Agilent Technologies). Average RIN was 8.0 in sow samples and 7.2 in piglet samples. Complementary DNA was generated from total RNA by using a SuperScript™ IV VILO™ Master Mix cDNA Synthesis Kit with ezDNase enzyme (Invitrogen) according to the manufacturer's instructions. Reverse transcription products were stored at -80 °C until PCR was performed. Considering the quantity and quality criteria, 151 RNA samples from sows' (n=18-20 per mitogen condition and per sow treatment) and 319 RNA samples from piglets' (n=7-12 per mitogen condition and per piglet treatment) whole blood cultures were used for quantitative PCR analyses.

To measure mRNA levels, high-throughput real-time PCR amplifications were performed using the SmartChip Real-Time PCR system (Wafergen Inc., USA) available at the EcogenO Platform (Human and Environmental Genomics (GEH), Rennes, France). The primer pairs used for real-time PCR have been designed from porcine sequences available in Ensembl or NCBI databases using Primer Express® v3.0 software (Applied Biosystems). The primers used are listed in Supplementary Table S1. The amplifications reactions were performed in duplicate for each sample and primer set combination using LightCycler 480 SYBR Green 1 Master (Roche Diagnostics, Meylan, France) with a final cDNA concentration of 1ng/mL and a primer concentration of 500nM dispensed using the WaferGene SmartChip Multisample Nanodispenser as already described (Gondret et al., 2021). Specificity of the amplification products was checked by dissociation curve analysis. The *hypoxanthine phosphoribosyltransferase 1 (HPRT1)*, *peptidylprolylisomerase A (PPIA)* and *TATA box binding protein (TBPI)* genes were evaluated as potential housekeeping genes. As stated by the GeNorm algorithm (<https://genorm.cmgg.be/>), the most stable genes among the 3 housekeeping genes tested were PPIA and TBPI, and were used to calculate the normalization factor (NF). For each gene, the normalized expression level N was calculated according to the formula:  $N = E - \Delta Cq \text{ (sample-calibrator)} / NF$  where E was calculated from the slope of

calibration curve, C<sub>q</sub> was the quantification cycle, and the calibrator was a newly generated biological sample constituted by the pool of the 71 samples. For all the genes studied, E was between 1.81 and 2.10.

***Colostrum and milk composition.*** Dry matter, ash, gross energy, crude protein, fat, and lactose were assayed in colostrum and milk as previously described by Loisel et al. (2013). Immunoglobulin G concentrations were assayed in colostrum using the same method as for plasma IgG except for the antibodies used due to a supply shortage from the supplier. The coating and the labeled antibodies (goat anti-pig IgG, Ref. A100-104A and HRP-labeled goat anti-pig IgG, Ref. A100-104P, respectively) were provided by Bethyl Laboratories, Montgomery, TX, USA). Colostrum samples were diluted at 1:1,000,000. Concentrations of IgA were assayed using a sandwich ELISA method using goat anti-pig IgA (Bethyl Laboratories, Ref. A100-102A) diluted at 10 mg/L as capture antibody, HRP-labeled goat anti-pig IgA (Bethyl Laboratories, Ref. A100-102P) diluted 1:50,000 as detecting antibody and native porcine IgA (ranging from 400 to 3.125 ng/mL, Alpha Diagnostic International, San Antonio, TX, USA, Ref. 20017-4-1) as standard. Samples were diluted at 1:100,000 (colostrum samples) or at 1:50,000 (milk samples) in dilution buffer (TBS with 0.05% Tween 20 and 1% BSA) The intra- and interassay CV were 8.7 and 4.8% for IgG and 6.2 and 8.1 % for IgA, respectively.

Mammary epithelium integrity was estimated by measuring the Na<sup>+</sup>:K<sup>+</sup> ratio in colostrum and milk samples. A 200- $\mu$ L sample of colostrum and a 500- $\mu$ L sample of milk were used for total milk Na<sup>+</sup> and K<sup>+</sup> analysis by inductively coupled plasma optical emission spectroscopy (ICP-OES 5110 Agilent Technology, Les Ulis, France). Colostrum and milk samples were first 100 and 40 times diluted in H<sub>2</sub>O, respectively. Then, 2.5 mL of 0.01% Triton X100 (Sigma Aldrich) and 7.5 mL of 65% nitric acid were added. The samples were then

completed to 50 mL with H<sub>2</sub>O. The analyses were performed in duplicate according to manufacturer instructions using calibration standards for ICP-OES Certipur Potassium and Sodium 1000 µg/mL (Agilent Technology, Les Ulis, France) and a standard milk sample ERM-BD151 (European Reference Materials, milk sample with guaranteed contents, European Commission Directorate-General JRC - Joint Research Centre Brussels, Belgium).

Haptoglobin in colostrum and milk samples was assayed using a commercial kit (Phase<sup>TM</sup> Haptoglobine assay kit, Tridelta Development Ltd) adapted to an automated multiparameter analyzer (Kone Instrument). Colostrum and milk samples were defatted by centrifugation before the analyses.

### ***Statistical analyses***

All statistical analyses were performed in the open-source environment R version 4.1.1. (R Development Core Team, Vienna, Austria). The number of piglets per litter was analyzed with the `glmer` function of the `lme4` package using a Poisson distribution and a log-transformation as the link function. All other data were analyzed using linear mixed-effect models using the `lmer` function from the `lme4` package.

For sow and litter performance, sow lymphocyte PI, sow gene expression in whole blood cell, and colostrum and milk composition, the sow or her litter was considered as the statistical unit and the models included the sow group (sCTRL or sTRT) as main effect and the batch (1, 2, 3, or 4) as random effect. For the indicators of the sow metabolic, immune, inflammatory, and oxidative status, the sow was considered as the statistical unit and the models included the sow group (sCTRL or sTRT), the day of sampling (DG94, DG112 and DL26) and the sow group x day of sampling interaction as main effects and the batch as random effect. For piglet growth performance, the piglet was considered as the statistical unit and the models included

the group of the sow (sCTRL or sTRT), the group of the piglet (pCTRL or pTRT) and the interaction between both groups as main effects. For the BW on DPW35, the value of the weaning BW was added to the model as a covariate. The sow and the batch were included as random effects. For the physiological parameters measured on the first pair of piglets during the neonatal period, the models included the group of the sow as main effects and the batch as random effect for parameters measured on DL3. For physiological parameters measured on DL14, the model included the group of the sow, the group of the piglet and the interaction between the two factors as main effects and the batch as random effect. The value of the same parameters measured on DL3 was added to the model as a covariate. Finally, for blood parameters measured on the second pair of piglets during the peri-weaning period, the models included the group of the sow, the group of the piglet, the day of sampling (DL25 or DPW5) and the interaction between the three factors as main effects and a batch random effect. The triple interaction not being significant, the effects of sow and piglet treatments were then analysed for each day separately. Data that were not normally distributed were submitted to a  $\log_{10}$  transformation to fit normal distribution. The assumption of normality of residuals was checked for each model through visual inspection of the QQ-plot of the residuals. All data were expressed as estimated marginal means  $\pm$  SEM. The statistical significance threshold was set at  $P \leq 0.05$ , and the trend-level significance was defined as  $0.05 < P \leq 0.10$ .

## **Results**

### ***Sow performance and litter characteristics***

From the beginning (on DG93) to the end (on DL28) of the experiment, sows' BW and backfat thickness did not differ between the two groups ( $P > 0.10$ , Supplementary Table S2). The supplementation did not affect the average daily feed intake of sows ( $P > 0.10$ ,

Supplementary Table S2). The BW and backfat thickness loss during lactation did not differ between sCTRL and sTRT sows ( $P > 0.10$ , Supplementary Table S2) and averaged  $-13.9 \pm 3.34$  kg and  $-3.2 \pm 0.71$  mm, respectively. Reproductive performance of sows (litter size, litter weight, and piglet mortality rate) did not differ between sow treatment groups ( $P > 0.10$ , Supplementary Table S3).

### ***Sow metabolic, immune, inflammatory and oxidative status***

The interaction between the sow group and the day of sampling was not significant ( $P > 0.10$ ). Hence the effect of sow group (sCTRL vs. sTRT) is presented for each day of sampling separately in Table 1 and Supplementary Tables S4 and S5.

Before the beginning of the sow supplementation (i.e., on DG94), no difference in the indicators of sows' metabolic, immune, inflammatory and oxidative status were observed between sCTRL and sTRT sows ( $P > 0.10$ ). On DG112, no treatment effect was observed ( $P > 0.10$ ) on plasma concentrations of metabolites, haptoglobin, BAP, dROM and the OSI, and the number of total white blood cells. However, sTRT sows had a lower number of lymphocytes than sCTRL sows (-12%,  $P < 0.05$ , See Supplementary Table 4). On DL26, the supplementation of sows did not affect significantly the plasma concentrations of the indicators of their metabolic, immune, inflammatory and oxidative status ( $P > 0.10$ ), except for NEFA whose concentrations were lower in sTRT compared to sCTRL sows ( $967.1 \pm 129.84$  vs.  $1239.7 \pm 129.77$   $\mu\text{M}$ ,  $P < 0.05$ , Table 1). The PI of lymphocytes and the expression level of TLR and cytokines in whole blood cell cultures did not differ between sow groups ( $P > 0.10$ , see Supplementary Tables S4 and S5).

### ***Colostrum and milk composition***

The composition of colostrum and milk on DL6 was not influenced by the sow supplementation (Table 2). On DL26, milk of sTRT sows contained 15% less fat ( $P < 0.05$ ) and 38% less haptoglobin ( $P < 0.01$ ) and tended to contain less dry matter (-9%,  $P = 0.07$ ) and gross energy (-11%,  $P = 0.06$ ) compared to the milk of sCTRL sows (Table 2). Milk of sTRT sows also tended to be less concentrated in  $\text{Na}^+$  ( $372 \pm 71.5$  vs.  $418 \pm 71.4$  mg/kg,  $P = 0.08$ ) whereas the  $\text{K}^+$  concentration was similar in milk of sows from both treatments, resulting in a tendency towards a lower  $\text{Na}^+:\text{K}^+$  ratio in the milk of sTRT sows ( $0.54 \pm 0.087$  vs.  $0.61 \pm 0.087$ ,  $P = 0.10$ , Table 2).

### ***Piglet growth performance***

Piglets born from sTRT and sCTRL sows had a similar average BW at birth, on DL6, at weaning and on DPW35 and a similar ADG from birth to DPW35 ( $P > 0.10$ , Table 3). However, control piglets born from supplemented sows (sTRT-pCTRL piglets) had a lower ADG from birth to the end of the post-weaning period compared to control piglets born from non-supplemented sows (sCTRL-pCTRL piglets,  $P < 0.05$ , Table 3).

Regardless of sow treatment, pTRT piglets were lighter on DL6 ( $2.51 \pm 0.050$  vs.  $2.67 \pm 0.050$  kg,  $P < 0.01$ ) and had a lower ADG from birth to DL6 ( $171 \pm 6.9$  vs.  $190 \pm 6.9$  g/d,  $P < 0.01$ ) than pCTRL piglets (Table 3). The BW at weaning did not differ between pCTRL and pTRT piglets but a significant interaction between the sow and the piglet treatment groups was observed on the ADG from DL6 to weaning: pTRT piglets had higher ADG only when they were born from sTRT sows ( $0.325 \pm 0.0084$  vs.  $0.298 \pm 0.0085$  kg/d,  $P < 0.05$ , Table 3). The pTRT piglets were heavier on DPW35 ( $25.3 \pm 1.76$  vs.  $24.3 \pm 1.76$  kg,  $P < 0.05$ ) and had a higher ADG from weaning to the end of the post-weaning period ( $0.431 \pm 0.0221$  vs.  $0.406 \pm 0.0220$  kg/d,  $P < 0.05$ ) compared to pCTRL piglets.

### ***Piglet immune status during the perinatal period***

On DL3, plasma concentrations of IgG and IFN- $\alpha$  did not differ between piglets born from sTRT and sCTRL sows ( $P > 0.10$ , Table 4). On DL14, plasma concentrations of IgG and IgM were not influenced by the sow and the piglet supplementation ( $P > 0.10$ ). However, there was an interaction between the maternal and the piglet supplementation in that sTRT-pCTRL piglets ( $36.9 \pm 8.34$  U/mL) had lower plasma concentration of IFN- $\alpha$  than both sCTRL-pCTRL ( $62.5 \pm 8.66$  U/mL) and sTRT-pTRT ( $61.1 \pm 8.88$  U/mL) piglets, whereas plasma concentration of IFN- $\alpha$  of sCTRL-pTRT ( $51.0 \pm 8.34$  U/mL) piglets was intermediate ( $P < 0.05$ , Table 4).

### ***Piglet immune, inflammatory and oxidative status around weaning***

The effect of weaning on piglet immune, inflammatory and oxidative status was assessed by comparing the value of plasma indicators 3 d before (DL25) and 5 d after (DPW5) weaning. Weaning induced a 22% and a 100% increase in the number of lymphocytes and neutrophils, respectively ( $P < 0.001$ ). Accordingly, total white blood cell numbers increased by 46% after weaning (from  $9.3 \pm 0.77$  on DL25 to  $13.6 \pm 1.12 \times 10^3/\text{mm}^3$  on DPW5,  $P < 0.001$ ). Weaning also modified the relative proportions lymphocytes and neutrophils: lymphocyte percentage was lower ( $63.0 \pm 1.29\%$  vs.  $54.1 \pm 1.29\%$ ,  $P < 0.001$ ) whereas neutrophil percentage was higher ( $24.6 \pm 2.30$  vs.  $33.4 \pm 2.30\%$ ,  $P < 0.001$ ) on DPW5 compared to DL25. After weaning, IgG concentrations were 28% lower ( $P < 0.001$ ) whereas IgM and haptoglobin concentrations were 44% and 563% higher ( $P < 0.001$ ), respectively compared to before weaning. Finally, a BAP decreased (-5%,  $P < 0.001$ ) and dROM increased (+47%,  $P < 0.001$ ), resulting in a 54% increase of the OSI ( $P < 0.001$ ).

The effect of the sow (sCTRL vs. sTRT) and piglet (pCTRL vs. pTRT) group on the indicators of piglets' immune, inflammatory and oxidative status are presented in Tables 5 (plasma concentrations of immune, inflammatory and oxidative status indicators and lymphocyte proliferation index) and 6 (level of gene expression in whole blood cell cultures). Maternal supplementation had no effect on the indicators measured in piglets before weaning on DL25 and after weaning on DPW5 except for the immune cell response to TLR agonist stimulation. Cells stimulated with LTA collected on DL25 in piglets born from sTRT sows expressed more IL-10 than those collected in piglets born from sCTRL sows ( $P < 0.05$ ). Unstimulated cells collected on DPW5 in piglets born from sTRT sows tended to express less TLR4 than those of piglets born from sCTRL sows ( $P = 0.08$ ).

Irrespective of the sow group, before weaning, pTRT piglets had a lower percentage of neutrophils ( $P < 0.05$ ) and tended to have a higher percentage of lymphocytes and a lower OSI ( $P = 0.07$  and  $P = 0.06$ , respectively, Table 5). Before weaning, unstimulated cells from pTRT piglets expressed more IL-1 $\beta$  mRNA than those of pCTRL piglets ( $P < 0.05$ ). In the LPS-stimulated conditions, cells from pTRT piglets expressed more IL-1 $\beta$  ( $P < 0.001$ ), IL-10 ( $P < 0.05$ ) and TNF- $\alpha$  ( $P < 0.05$ ) mRNA than those of pCTRL piglets. In the LTA-stimulated condition, the expression of IL-1 $\beta$  was higher ( $P < 0.05$ ) in cells from pTRT piglets compared with those of pCTRL piglets. A significant interaction between the sow and the piglet groups was observed on the plasma concentrations of IgM: pTRT piglets had higher plasma IgM concentration than pCTRL piglets, only in piglets born from sTRT sows ( $0.70$  vs  $0.53 \pm 0.077$  mg/mL,  $P = 0.05$ , Table 5).

After weaning, piglet supplementation had no significant effects on the indicators of the inflammatory and oxidative status ( $P > 0.10$ , Table 5). It also had little effects on immune cell function in vitro: the mRNA expression of TNF- $\alpha$  in unstimulated conditions and of INF- $\alpha$  in ODN-stimulated conditions tended to be lower ( $P = 0.08$  and  $P = 0.09$ , respectively) whereas



the mRNA expression of IL-10 in LPS-stimulated conditions tended to be higher ( $P = 0.10$ ) in cells from pTRT piglets compared to those of pCTRL piglets (Table 6). Finally, the expression of TNF- $\alpha$  in LPS-stimulated cells from pTRT piglets was higher, only in piglets born from sCTRL sows ( $2.63 \pm 0.595$  vs  $1.55 \pm 0.595$ ,  $P < 0.05$ ).

## Discussion

The present experiment consisted in studying the impact of dietary supplementations on the response of piglets to weaning. The supplementations administered to sows and piglets were blends of plant extracts. The effects observed could be attributed to a variety of bioactive molecules, which have been selected because of its potential immune system-promoting, anti-inflammatory, antioxidative, antibacterial or anticoccidial properties suggested by *in vitro* or *in vivo* experiments. Moreover, each component of the blends might also interact with each other. Therefore, an effect cannot be attributed to one specific component of the blends. Weaning the piglets in challenging conditions successfully induced an immune, inflammatory and oxidative response. This was demonstrated by the increase in the immune cell numbers, haptoglobin concentration, and OSI in piglets' plasma 5 d after compared to 3 d before weaning. Weaning conditions similar to those of the present study have already been shown to challenge piglet health (Buchet et al., 2017; Le Floc'h et al., 2022). These findings are also consistent with the results of previous studies pointing out an activation of the immune system (Kojima et al., 2008; Salak-Johnson and Webb, 2018; de Groot et al., 2021), an inflammatory reaction (McCracken et al., 1995; Sauerwein et al., 2005; Pastorelli et al., 2012), and a degradation of the oxidative status (Zhu et al., 2012; Buchet et al., 2017) of piglets in response to weaning. The challenging weaning conditions were applied in our study to reveal the potential beneficial effects of the maternal and piglet supplementations with our combination of plant extracts on the physiological status of piglets and in turn on their ability to face the weaning stress. This

experimental strategy was applied since it has been hypothesized that robustness is revealed in challenging environments (Friggens et al., 2017). After weaning, an increase in IgM and a decrease in IgG concentrations were also observed but these variations were more likely related to piglet age than to weaning. Indeed, the IgG concentration was observed to decrease continuously from DL3 to DPW5. This decrease in IgG is likely explained by the progressive degradation and disappearance over time of maternal IgG acquired by colostrum (Klobasa et al., 1981). The increase of IgM, which are produced during primary antibody responses, reflects the progressive development of piglet's own antibody repertoire upon environmental antigenic stimulation (Hervé et al., 2022).

Findings of the present study demonstrate that providing the combination of plant extracts to sows during late gestation and lactation influenced their physiology and the composition of their colostrum and milk. First, sows supplemented with our combination of plant extract had a lower number of lymphocytes in plasma on DG112 compared to non-supplemented sows. Variations in blood lymphocyte numbers can result from changes either in the hematopoietic process and cell survival, in relation to pathological or infectious situations, either in cell redistribution among the body, due to diurnal rhythms or hormonal stress-related fluctuations (Lange et al., 2022). Variation within the normal range, as in the present case, are uneasy to interpret functionally and may reflect subtle differences in health status of the animals, as observed in apparently healthy weaned pigs exposed to high or low pathogen burden in their living environment (Hervé et al., 2022). Our preventive strategy based on plant extract supplementation failed to improve the immune quality of colostrum and milk assessed by IgG and IgA concentrations. Nevertheless, milk of supplemented sows had a lower concentration of the acute phase protein haptoglobin than non-supplemented sows at the end of lactation. Haptoglobin in milk can originate from the transfer of haptoglobin from the plasma of the sow to the mammary secretions but can also have a local origin since the mammary gland is an

extrahepatic source of haptoglobin (Hiss et al., 2004). In cows, haptoglobin concentrations in milk may be indicators of udder health (Grönlund et al., 2005). Less haptoglobin in milk may thus indicate that supplemented sows had a healthier udder in late lactation than control sows. Maternal haptoglobin can be transferred to the piglets via the colostrum and exert systemic immunomodulating actions (Hiss-Pesch et al., 2011). To our knowledge, however, there is no report on the potential impact of haptoglobin originating from mature milk.

At the end of lactation, the milk produced by the supplemented sows contained less fat than the milk of the control sows. The fat content in milk is influenced by diet, notably fat and fiber content, and mobilization of body lipids (Hurley, 2015). Here, sows were fed the same diet, and they consumed the same average amount of feed daily. The supplemented sows had lower plasma concentrations of NEFA than control sows on DL26, which suggests that they mobilized less body lipid reserve at the end of lactation. Nevertheless, if supplemented and control sows did diverge in lipid mobilization, the difference must have been moderate, as it had no impact on the variation in backfat thickness over the whole lactation. There are few reports concerning the effect of sow supplementation with plant extracts on the nutritional composition of their colostrum and milk. The supplementation of sows with oregano essential oil containing carvacrol and thymol reduced milk fat content when provided during lactation (Ariza-Nieto et al., 2011) or had no significant effect on colostrum and milk nutrient composition when provided through gestation and lactation (Tan et al., 2015). The supplementation of sows with a blend of essential oils whose main bioactive compounds are eucalyptol, p-cymene, linalool, anethole, and thymol during gestation and lactation increased colostrum protein content and milk fat content (Reyes-Camacho et al., 2020). Therefore, the mechanisms underlying the effect of sow supplementation on milk fat content remains to be investigated. In the present study, the lower fat content in the milk of TRT sows did not affect average piglet growth from birth to weaning.

The maternal supplementation had few effects on the indicators of the immune, inflammatory and oxidative status of piglets during the suckling period and around weaning. Indeed, piglets born from sTRT and sCTRL sows had similar plasma IgG concentrations on DL3, DL14 and around weaning. This is consistent with the lack of effect of maternal treatment on colostrum IgG content. The plasma concentration of IFN- $\alpha$  has been used as an indicator of innate immune response to viral infection. In our study, plasma IFN- $\alpha$  concentrations of piglets on DL2 and DL14 were much lower than those reported in infected pigs (Gonzalez et al., 2010; Annamalai et al., 2015) and tended to decrease with age. Detectable and age-decreasing concentrations of IFN- $\alpha$  have also been described in piglets around weaning age (Hervé et al., 2022). These results and ours suggest that elevated concentrations of IFN- $\alpha$  might occur in neonatal piglets independently from any viral infection and decrease progressively in the following weeks. On DL14, pCTRL piglets born from sTRT sows had lower plasma concentrations of IFN- $\alpha$  compared to pCTRL piglets born from sCTRL sows. However, we have no explanation for the interaction between the maternal and the piglet supplementation and the lack of study reporting basal concentration of IFN- $\alpha$  in young pigs makes it difficult to conclude whether this effect was beneficial or detrimental for the piglets. The maternal supplementation had no effect on the indicators of the immune, inflammatory and oxidative status of piglets around weaning with the exception of a greater expression of IL-10 mRNA, in LTA-stimulated cells on whole blood cultures of piglets born from supplemented sows on DL25. Interleukin-10 is an anti-inflammatory cytokine, which can help to control excessive or terminate inflammatory responses. The decrease in the expression of IL-10 mRNA and not of other genes in response to LTA, suggests a specific down-regulation of this cytokine and not a global lower sensibility of cells to LTA. However, because this is the only effect of maternal supplementation among all the indicators of piglets' immune status measured *in vivo* and *in vitro*, this result should not be over-interpreted. Moreover, the maternal supplementation did

not improve the growth performance of piglets (ADG and BW) during the suckling period and around weaning. All together, our results show that supplementing sows with our combination of plant extracts did not improve the robustness of piglets during the suckling and post-weaning period.

Contrary to the maternal supplementation, the administration of plant extracts as a single dose of the liquid solution directly to piglets influenced their performance and physiological status. The treatment transiently impaired piglet ADG and BW on DL6. This reduced growth performance is probably due to the high doses of essential oils as already suggested in poultry (Alleman et al., 2013). However, the supplementation of piglets improved their post-weaning growth performance probably by improving their immune and antioxidant status before weaning. Indeed, before weaning, the supplementation of piglets with the liquid solution of plant extracts tended to reduce the OSI, suggesting an improvement of the oxidative status. The piglet supplementation also decreased the proportion of neutrophils among leukocytes. This result may suggest a lower activation of the innate immune system of pTRT piglets. The components of the liquid solution used for piglets' supplementation were chosen for their well-known antimicrobial properties when used alone or in combination (Sivropoulou et al., 1996; Zhou et al., 2007; Luís et al., 2016). The supplementation of piglets may have modified the bacterial pressure in their intestine resulting in the lower activation of their innate immune system. Investigations on the piglet gut microbiota might confirm this hypothesis. We also investigated the piglet white blood cells function by studying their ability to respond *in vitro* to molecules mimicking bacterial and viral infections. To do so, we analyzed the level of gene expression of TLR-2, -4, and -9 in whole blood cells in response to the stimulation of these receptors with their specific agonists. These three TLR are specialized in the recognition of Gram-positive bacteria, Gram-negative bacteria, and DNA-viruses, respectively (Kawai and Akira, 2011). The binding of microbial ligands to TLR induces the release of cytokines,

responsible for the initiation and regulation of the immune response necessary to eliminate pathogens, among which IL-1 $\beta$ , IL-10, TNF- $\alpha$  (for TLR-2 and -4) and IFN- $\alpha$  (for TLR-9) (Kawai and Akira, 2011). The direct supplementation of piglets with plant extracts did not affect the gene expression of TLR but increased the gene expression of pro- and anti-inflammatory cytokines in unstimulated (IL-1 $\beta$ ), LPS-stimulated (IL-1 $\beta$ , IL-10, TNF- $\alpha$ ) and LTA-stimulated (IL-1 $\beta$ ) conditions, suggesting that the immune cells of supplemented piglets have a better ability to produce an inflammatory response in response to bacterial signals, and especially to Gram negative bacteria signals (LPS). We also observed an interaction of maternal and piglet plant extract treatment on piglets' immune system. Indeed, on DL25, pTRT piglets born from sTRT sows had higher plasma concentrations of IgM compared to pCTRL piglets born from sTRT sows. This greater IgM synthesis might result from a higher aptitude of the immature immune system of piglets to produce primary humoral responses. Interestingly, the sTRT-pTRT piglets also had higher plasma concentrations of IFN- $\alpha$  on DL14 compared to sTRT-pCTRL piglets. The higher plasma concentrations of these two indicators suggests a greater activation of the immune system of these piglets. However, the mechanisms leading to such an interaction between maternal and piglet treatment are unclear. The effects of the direct supplementation of piglets with the liquid solution of plant extracts on the in vivo and in vitro indicators of the immune and oxidative indicators were no longer observed after weaning. The reason is probably that the impact of weaning on the measured parameters had overwhelm these effects. Nevertheless, the direct supplementation of piglets with the blend of essential oils had a positive impact on their growth performance during the post-weaning period as demonstrated by heavier piglets at the end of the post-weaning period and the higher post-weaning average daily gain in supplemented piglets. The findings of the present study suggest that the greater growth performance of supplemented piglets during the post-weaning period might be associated with the limitation of their oxidative stress and the improvement of their immune status before

weaning. Nevertheless, an impact of piglet supplementation on other functions, such as protein and energy metabolisms, and feed conversion, cannot be ruled out.

## **Conclusion**

The findings of the present study show that the preventive strategy based on the maternal supplementation with two blends of plant extracts failed to improve piglets' robustness at weaning as shown by the absence of effect on the physiological status and performance of piglets born from these sows. The supplementation of 3-d old piglets with the liquid solution of plant extracts did improve their growth performance during the post-weaning period, suggesting that this preventive strategy improved their robustness at weaning. This beneficial effect could be linked with the improvement of the immune function and the oxidative status of piglets before weaning.

## **Supplementary data**

Supplementary data are available at Journal of Animal Science online.

## ***Conflict of interest statement***

There is no conflict of interest to be declared. However, A. Greuter and L. Hugonin are employees of IDENA, the company who provided the plant extract and essential oil blends for this experiment.

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**Table 1.** Plasma concentrations of metabolites in sows supplemented with plant extracts (sTRT group) or not (sCTRL group). Plasma samples were collected on day 94 (DG94) and 112 of gestation (DG112) and on day 26 of lactation (DL26).

Item	Sow group		SEM	<i>P</i> -value
	sCTRL	sTRT		
<b>On DG94 (n = 59)</b>				
Glucose, mg/L	813.1	816.2	32.96	0.91
Lactate, $\mu$ M	2571	2732	188.4	0.54
NEFA, $\mu$ M	92.6	114.0	121.04	0.85
Creatinine, mg/L	22.8	22.6	0.49	0.77
Urea, mg/L	198.2	198.8	10.12	0.96
<b>On DG112 (n = 59)</b>				
Glucose, mg/L	763.5	729.4	33.14	0.24
Lactate, $\mu$ M	2034.7	1874.2	187.98	0.54
NEFA, $\mu$ M	716.5	828.0	120.95	0.32
Creatinine, mg/L	23.5	23.4	0.49	0.78
Urea, mg/L	165.8	167.5	10.11	0.90
<b>On DL26 (n = 46)</b>				
Glucose, mg/L	693.3	729.0	35.67	0.28
Lactate, $\mu$ M	1961.1	2290.6	216.40	0.27
NEFA, $\mu$ M	1239.7	967.1	129.84	<b>&lt; 0.05</b>
Creatinine, mg/L	18.6	18.0	0.55	0.37
Urea, mg/L	305.2	295.6	11.91	0.53

**Table 2.** Composition of colostrum and milk collected on day 6 (DL6) and day 26 of lactation (DL26) from sows supplemented with plant extracts (sTRT group) or not (sCTRL group).

Item	Sow group		SEM	P-value
	sCTRL	sTRT		
<b>Colostrum (n = 63)</b>				
Dry matter, %	27.6	26.8	0.85	0.31
Ash, %	0.66	0.67	0.024	0.73
Protein, %	16.58	15.97	0.460	0.20
Lipid, %	4.42	4.91	0.286	0.23
Lactose, %	3.68	3.71	0.446	0.87
Gross energy, kJ/g	6.64	6.43	0.183	0.41
Na <sup>+</sup> , mg/kg	722	752	28.6	0.46
K <sup>+</sup> , mg/kg	1377	1380	35.1	0.95
Na <sup>+</sup> :K <sup>+</sup> ratio	0.54	0.57	0.030	0.52
IgG, mg/mL	46.90	46.36	3.912	0.92
IgA, mg/mL	22.73	21.20	1.570	0.45
Haptoglobin, mg/mL	1.54	1.59	0.445	0.72
<b>Milk at DL6 (n = 64)</b>				
Dry matter, %	20.9	20.1	0.62	0.12
Ash, %	0.79	0.81	0.023	0.23
Protein, %	5.76	5.62	0.138	0.47
Lipid, %	8.28	7.55	0.318	0.11
Lactose, %	7.72	7.79	0.881	0.77
Gross energy, kJ/g	5.46	5.17	0.133	0.12
Na <sup>+</sup> , mg/kg	392	354	35.4	0.27
K <sup>+</sup> , mg/kg	1200	1243	93.8	0.37
Na <sup>+</sup> :K <sup>+</sup> ratio	0.34	0.29	0.031	0.21
IgA, mg/mL	4.44	4.59	0.380	0.75
Haptoglobin, mg/mL	0.29	0.20	0.043	0.16
<b>Milk at DL26 (n = 48)</b>				
Dry matter, %	26.8	24.5	3.43	0.07
Ash, %	0.97	0.93	0.043	0.12
Protein, %	6.85	6.42	0.456	0.12
Lipid, %	13.57	11.46	3.100	<b>&lt; 0.05</b>
Lactose, %	6.31	6.72	1.300	0.29
Gross energy, kJ/g	7.62	6.81	1.23	0.06
Na <sup>+</sup> , mg/kg	418	372	71.5	0.08
K <sup>+</sup> , mg/kg	692	695	55.0	0.93
Na <sup>+</sup> :K <sup>+</sup> ratio	0.61	0.54	0.087	0.10
IgA, mg/mL	6.23	6.11	0.910	0.85
Haptoglobin, mg/mL	0.61	0.38	0.11	<b>&lt; 0.01</b>

**Table 3.** Growth performance of piglets born from sows supplemented with plant extracts (sTRT group) or not (sCTRL group) and directly supplemented with the liquid plant extract solution (pTRT group) or not (pCTRL group).

Item	Sow group (SG) Piglet group (PG)	sCTRL		sTRT		SEM	P-value		
		pCTRL	pTRT	pCTRL	pTRT		SG	PG	SG x PG
<b>Average body weight, kg</b>									
At birth		1.43		1.51		0.052	0.14		
On day of lactation (DL) 6		2.65	2.56	2.69	2.45	0.073	0.67	< 0.01	0.24
At weaning		8.85	8.71	8.69	8.54	0.230	0.44	0.44	0.97
On day post-weaning 35		24.6	25.1	24.0	25.5	1.80	0.84	< 0.05	0.34
<b>ADG, kg/d</b>									
Birth-DL6		0.200	0.173	0.181	0.169	0.0087	0.13	< 0.01	0.27
DL6-weaning		0.314 <sup>ab</sup>	0.303 <sup>ab</sup>	0.298 <sup>a</sup>	0.325 <sup>b</sup>	0.0086	0.73	0.34	< 0.05
Post-weaning period		0.422	0.426	0.389	0.436	0.0240	0.40	< 0.05	0.09
Total		0.373 <sup>b</sup>	0.368 <sup>ab</sup>	0.341 <sup>a</sup>	0.374 <sup>b</sup>	0.0154	0.14	0.11	< 0.05

<sup>a,b</sup> Within a row, means without a common superscript letter differ ( $P \leq 0.05$ )

**Table 4.** Plasma concentrations of immunological indicators of piglets born from sows supplemented with plant extracts (sTRT group) or not (sCTRL group) and directly supplemented with the liquid plant extract solution (pTRT group) or not (pCTRL group) measured during the neonatal period.

Item <sup>1</sup>	Sow group (SG)		Piglet group (PG)		SEM	P-value		
	sCTRL	pCTRL	sTRT	pTRT		SG	PG	SG x PG
<b>On DL3 (n = 128)<sup>2</sup></b>								
IgG, mg/mL	29.67		27.39		2.261	0.33		
IFN- $\alpha$ , U/mL	111.0		186.4		66.57	0.16		
<b>On DL14 (n = 127)</b>								
IgG, mg/mL	8.46	8.66	8.11	8.10	0.940	0.40	0.86	0.83
IgM, mg/mL	0.27	0.29	0.31	0.29	0.028	0.26	0.70	0.28
IFN- $\alpha$ , U/mL	62.5 <sup>b</sup>	51.0 <sup>ab</sup>	36.9 <sup>a</sup>	61.1 <sup>b</sup>	8.88	0.36	0.46	< <b>0.05</b>

<sup>1</sup>Plasma samples were collected before (on day 3 of lactation, DL3) and after (on DL14) the administration of the liquid solution to piglets.

<sup>2</sup>On DL3, plasma sample were collected before the administration of the liquid solution. For this reason, only the effect of maternal supplementation has been studied.

<sup>a,b</sup> Within a row, means without a common superscript letter differ ( $P \leq 0.05$ )

**Table 5.** Plasma concentration of immunological, inflammatory and oxidative indicators of piglets born from sows supplemented with plant extracts (sTRT group) or not (sCTRL group) and directly supplemented with the liquid plant extract solution (pTRT group) or not (pCTRL group) measured during the peri-weaning period.

Item <sup>1</sup>	Sow group (SG)	sCTRL		sTRT		SEM	P-value		
	Piglet group (PG)	pCTRL	pTRT	pCTRL	pTRT		SG	PG	SG x PG
<b>On DL25 (n = 95)</b>									
White blood cells, 10 <sup>3</sup> /mm <sup>3*</sup>		9.2	9.6	9.3	9.2	1.09	0.68	0.79	0.61
Lymphocytes, 10 <sup>3</sup> /mm <sup>3*</sup>		5.6	6.1	5.8	5.9	0.62	0.95	0.24	0.55
Neutrophils, 10 <sup>3</sup> /mm <sup>3*</sup>		2.3	2.2	2.3	2.0	0.39	0.46	0.31	0.75
Lymphocytes, %		61.1	63.8	62.5	64.8	2.01	0.37	0.07	0.89
Neutrophils, %		26.4	23.8	25.4	22.7	2.42	0.39	< <b>0.05</b>	0.94
IgG, mg/mL		4.29	4.18	4.01	4.31	1.120	0.91	0.87	0.73
IgM, mg/mL		0.65 <sup>ab</sup>	0.56 <sup>ab</sup>	0.53 <sup>a</sup>	0.70 <sup>b</sup>	0.080	0.85	0.50	< <b>0.05</b>
Proliferation index <sup>2*</sup>		6.96	4.60	6.47	6.71	1.246	0.22	0.13	0.07
Haptoglobin, mg/mL		0.64	0.57	0.25	0.29	0.251	0.14	0.95	0.82
BAP, μM Eq vitamin C		2764	2844	2783	2788	48.7	0.58	0.21	0.27
dROM, CarrU		712	649	674	624	39.0	0.38	0.12	0.85
OSI, CarrU/μM Eq vitamin C		0.26	0.23	0.24	0.22	0.016	0.44	0.06	0.60
<b>On DPW5 (n = 95)</b>									
White blood cells, 10 <sup>3</sup> /mm <sup>3*</sup>		13.2	14.0	13.6	13.6	1.02	0.99	0.53	0.56
Lymphocytes, 10 <sup>3</sup> /mm <sup>3*</sup>		6.9	7.2	7.3	7.4	0.64	0.38	0.48	0.70
Neutrophils, 10 <sup>3</sup> /mm <sup>3*</sup>		4.3	4.6	4.3	4.3	0.45	0.69	0.70	0.65
Lymphocytes, %		53.7	52.6	54.9	55.3	2.09	0.29	0.86	0.69
Neutrophils, %		33.8	34.2	33.1	32.5	2.93	0.45	0.94	0.78
IgG, mg/mL		3.46	2.83	3.00	2.87	0.552	0.48	0.21	0.42
IgM, mg/mL		0.93	0.86	0.85	0.91	0.073	0.84	0.96	0.20
Proliferation index <sup>*</sup>		6.50	6.13	6.94	5.72	1.711	0.99	0.29	0.57
Haptoglobin, mg/mL		2.80	3.08	2.97	2.55	0.277	0.44	0.77	0.15
BAP, μM Eq vitamin C		2669	2642	2630	2652	53.2	0.57	0.92	0.34
dROM, CarrU		1022	974	984	934	44.4	0.25	0.15	0.96
OSI, CarrU/μM Eq vitamin C		0.38	0.37	0.37	0.35	0.014	0.30	0.16	0.76

\*Data submitted to a log10 transformation prior to statistical analyses.

<sup>1</sup>Plasma samples were collected on day 25 of lactation (DL25) and 5 d post-weaning (DPW5).

<sup>2</sup>The proliferation index in response to Concanavalin A stimulation allowed measuring the capacity of lymphocytes to proliferate in vitro.

<sup>a,b</sup> Within a row, means without a common superscript letter differ ( $P \leq 0.05$ )



**Table 6.** Relative expression of target genes quantified by real-time PCR in whole blood cell cultures from piglets born from sows supplemented with plant extracts (sTRT group) or not (sCTRL group) and directly supplemented with the liquid plant extract solution (pTRT group) or not (pCTRL group).

Item <sup>1</sup>	Sow group (SG)		sCTRL		sTRT		SEM	<i>P</i> -value		
	Piglet group (PG)		pCTRL	pTRT	pCTRL	pTRT		SG	PG	SG x PG
<b>On DL25</b>										
<b>Med.</b>										
IFN- $\alpha$			1.60	1.28	1.20	1.29	0.222	0.23	0.52	0.20
IL-1 $\beta$			0.07	0.08	0.05	0.09	0.016	0.59	< <b>0.05</b>	0.29
IL-10			0.12	0.14	0.18	0.14	0.028	0.21	0.97	0.19
TNF- $\alpha$			0.42	0.42	0.48	0.54	0.080	0.16	0.66	0.62
TLR2			0.39	0.44	0.45	0.42	0.050	0.60	0.75	0.26
TLR4			0.69	0.72	0.69	0.69	0.088	0.80	0.89	0.80
TLR9			0.86	0.80	0.82	0.91	0.197	0.90	0.80	0.31
<b>LPS</b>										
IL-1 $\beta$			2.52	4.11	2.75	5.60	0.976	0.23	< <b>0.001</b>	0.50
IL-10			1.25	1.96	1.24	1.84	0.333	0.92	< <b>0.05</b>	0.99
TNF- $\alpha$			1.51	2.13	1.63	2.36	0.463	0.56	< <b>0.05</b>	0.92
TLR4			1.15	1.49	1.30	1.49	0.324	0.65	0.13	0.64
<b>ODN</b>										
IFN- $\alpha$			1.07	0.98	1.31	0.73	0.318	0.83	0.13	0.26
TLR9			0.41	0.36	0.36	0.34	0.089	0.59	0.60	0.85
<b>LTA</b>										
IL-1 $\beta$			0.21	0.30	0.14	0.44	0.207	0.98	< <b>0.05</b>	0.25
IL-10			0.22	0.20	0.29	0.35	0.068	< <b>0.05</b>	0.79	0.43
TNF- $\alpha$			0.67	0.69	0.67	0.92	0.132	0.28	0.20	0.28
TLR2			0.57	0.51	0.52	0.54	0.090	0.82	0.65	0.37
<b>On DPW5</b>										
<b>Med.</b>										
IFN- $\alpha$			1.26	1.10	1.13	1.40	0.347	0.69	0.82	0.31
IL-1 $\beta$			0.05	0.04	0.04	0.05	0.008	0.78	0.61	0.29
IL-10			0.30	0.26	0.27	0.27	0.049	0.84	0.61	0.66
TNF- $\alpha$			0.55	0.51	0.63	0.46	0.062	0.96	0.08	0.26
TLR2			0.97	1.03	0.97	0.84	0.088	0.22	0.59	0.25
TLR4			1.18	1.10	1.02	0.98	0.089	0.08	0.50	0.82
TLR9			2.40	2.14	2.12	2.41	0.486	0.98	0.95	0.23
<b>LPS</b>										
IL-1 $\beta$			1.74	3.36	3.10	3.59	1.157	0.26	0.16	0.37
IL-10			2.60	4.16	3.69	3.81	0.642	0.38	0.10	0.14
TNF- $\alpha$			1.55 <sup>a</sup>	2.63 <sup>b</sup>	2.30 <sup>ab</sup>	1.91 <sup>ab</sup>	0.593	0.84	0.35	0.05
TLR4			1.72	2.11	1.95	2.07	0.272	0.66	0.26	0.53
<b>ODN</b>										

IFN- $\alpha$	1.38	1.02	1.19	0.92	0.219	0.43	0.09	0.90
TLR9	0.69	0.75	0.70	0.66	0.113	0.60	0.89	0.46
<b>LTA</b>								
IL-1 $\beta$	0.24	0.20	0.26	0.23	0.104	0.68	0.62	0.87
IL-10	0.71	0.61	0.74	0.64	0.186	0.78	0.37	0.99
TNF- $\alpha$	0.78	0.83	1.02	0.74	0.156	0.63	0.45	0.24
TLR2	1.43	1.53	1.46	1.18	0.173	0.23	0.45	0.16

<sup>1</sup>Cells were collected from blood on day of lactation 25 (DL25) and 5 d post-weaning (5DPW) and cultivated for 20 h in medium alone (Med.) or in presence of lipopolysaccharide (LPS), a CpG oligonucleotide (ODN) or lipoteichoic acid (LTA).

<sup>a,b</sup> Within a row, means without a common superscript letter differ ( $P \leq 0.05$ )