Fermented Rye With *Agaricus Subrufescens* and Mannan-rich Hydrolysate Based Feed Additive to Modulate Post-weaning Piglet Immune Response

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Fermented rye with *Agaricus subrufescens* and Mannan-rich hydrolysate based feed additive to modulate post-weaning piglet immune response

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20   **Abstract**

21   *Background*

22   The process of weaning in piglets is often associated with an increased inflammation response in
23   the intestine and compromised intestinal integrity and morphology favoring a delay in intestinal
24   maturation and a predisposal to diseases. Research has shown the potential of different
25   nutritional strategies to reduce the production of pro-inflammatory cytokines, with the main goal
26   to manipulate health and performance of pigs. Promising examples of nutritional strategies are
27   fungal fermented products and their derivatives which are described to contain several
28   compounds that may play a role in gastrointestinal health and pathogenic bacteria control.
29   Products from *Agaricus subrufescens* mushroom are reported to contain prophylactic and
30   therapeutic properties including antimicrobial and immunomodulatory.

31   *Results*

32   This study analysed the post-weaning immune status in intestinal tissue and blood of piglets, with
33   the objective to evaluate the gastrointestinal health and immune modulation response induced by
34   a blend of mannan-rich hydrolyzed copra meal and fermented rye with *A. subrufescens*. The results
35   showed that in piglets post-weaning, the feed additive stimulates an immunomodulation effect
36   most evident at 15 days post-weaning, with lower expression of cytokines Interferon (IFN) γ ,
37   Interleukin (IL) 1 α , IL-1 β , IL-6, IL-8, IL-10 and Transforming Growth Factor (TGF) β in jejunum,
38   accompanied with an increase in peripheral blood mononuclear cells (PBMC) cytokine gene
39   expression of IL-1 β , IL-6, IL-8, IL-10, IL-12p35 (IL-12 α), IL-12p40 (IL-12 β), Tumor Necrosis
40   Factor (TNF) α , IFN- α , and TGF- β . In piglets fed the feed additive, the quantity of
41   Immunoglobulin (Ig) A producing cells in jejunum, ileum was reduced on day 15 and 30 post-

weaning, and on day 30 and 45 post-weaning in colon tissue. Intestinal histomorphology demonstrated a villus height reduction in jejunum and increase in ileum on day 15, while increased villous height in jejunum and ileum on day 30. Natural Killer (NK) cells were increased at day 15 post-weaning in the piglets fed the feed additive.

Conclusion

This study implies the potential of the blend including mannan-rich hydrolyzed copra meal and fermented rye with *A. subrufescens* on immune modulation in the intestine of post-weaning piglets.

Keywords

inflammation, cytokines, additive, intestine, piglets, weaning

Background

The gastrointestinal tract (GIT) is essential in the maintenance of health. The intestine main function is the uptake of nutrients while simultaneously form a physical barrier which should prevent toxic compounds and pathogens from crossing the intestinal mucosa and systemic circulation. Two major components of the intestinal barrier are the intestinal epithelium and the gut associated lymphoid tissue (GALT). This tissue has the challenging dual task of selectively absorbing nutrients from the intestinal lumen, while preventing microbial and toxins entry and infection.

Previous research has shown that the process of weaning in piglets is associated with an increased inflammation response in the intestine (1,2), and the potential negative effect of increased expression of inflammation markers on intestinal integrity (3,4), morphology of

intestinal structures such as villous length and crypt depth (1,5) and disruption of the microbiota (6,7), favoring a delay in intestinal maturation and a predisposal to diseases (8,9). Recent research by Pluske et al. (10) has shown the potential of different management measures around weaning such as implementing nutritional strategies to reduce intestinal pathogen load, increasing digestion and preventing production and activity of pro-inflammatory cytokines, all with the main goal to manipulate the immune system of pigs for improving performance, aiming to have an appropriate immune response for each specific circumstance, preventing to maximize the immune response (10).

Promising examples of nutritional strategies are metabolites derived from edible mushrooms. Fungal fermented products and their derivatives are described to contain several compounds that may play a role in gastrointestinal health and pathogenic bacteria control (11). Microbial enzymes produced by fungi during fermentation will degrade polysaccharides from feed material into indigestible and bioactive oligosaccharides (12). *A. subrufescens*, also known as *A. blazei murill* and/or the almond mushroom, is an edible mushroom, which grows naturally in Piedade, outside of São Paulo, Brazil. It contains high levels of biological response modulators, such as proteoglycans (13,14) and β -glucans (15), which are a heterogeneous group of polysaccharides present in cereal grains, fungal cell walls, seaweed, and algae (16). Products from *A. subrufescens* mushroom are reported to contain prophylactic and therapeutic properties including antimicrobial and immunomodulatory (11,17).

The gastro-intestinal immune system is geared towards tolerance, in contrary to the systemic immune system, and responds to intestinal content (microbiota, metabolites, feed components, etc), and this reaction can lead to tolerance (e.g. for commensal bacteria) or defense reactions (18). One of the strategies that the host utilizes to avoid an inflammatory response against the

microbiota is to use the intestinal barrier, including the mucous layer and immunoglobulin (Ig) A, an antibody isotype specialized in mucosal protection (19,20). Production of IgA is controlled by cytokine-producing T cells within the GALT and by cytokine released from the mucosa. Previous studies report changes in the expression of inflammatory cytokines in the intestine of humans and animals during enteric infection and intestinal inflammatory diseases (21–23). Both in vitro and in vivo investigations show that uncontrolled production of pro-inflammatory cytokines can influence gut integrity and epithelial functions, including permeability to macromolecules and transport of nutrients and ions (24). Interactions of immune cell populations, present in the epithelium of the intestine, and other components of the intestinal mucosa are essential in the maintenance of symbiosis with commensals and the defense against pathogens.

From a nutritional perspective, controlling early intestinal inflammation is certainly a challenge in managing post-weaning gut disorders and preventing intestinal inflammation due to pathogenic enteric organisms can be an important pillar to maintain the health of piglets. The objective of this study was to evaluate the gastrointestinal health and immune modulation response induced by a blend of mannan-rich hydrolyzed copra meal and fermented rye with *A. subrufescens* in weaned piglets.

Results

Growth Performance

Least squares means and standard errors for body weight (BW) and average daily gain (ADG) for control and FS treatment are given in Table 3. Average daily gain tended to increase in FS

treatment for day 0-15 ($P = 0.0788$). There was no statistical evidence for other performance differences between control and treatment group.

Intestinal histomorphometry

Data on small intestine villi characteristics are presented in Table 4. The villous length and crypt depth in jejunal tissue was lower on day 15 for FS compared to control ($P < 0.05$). At d 15 in ileal tissue the villous length tended ($P = 0.093$) to increase in FS compared to control. On day 30 an increase in jejunal tissue ($P < 0.05$) and a tendency in ileal tissue ($P = 0.093$) were observed in villous length for FS compared to control. In ileal tissue the V/C ratio was increased in FS compared to control on day 30 ($P < 0.05$).

Immunoglobulin A producing cells in intestinal tissues

Several differences were observed between dietary treatments for various intestinal tissues and timepoints. Compared to control, the average count of IgA producing cells on day 15 was lower ($P < 0.05$) in piglets fed the FS diet in jejunum and ileum, being not different in colon. On day 30, compared to control, the average count of IgA producing cells was lower ($P < 0.05$) in piglets fed with FS in jejunum, ileum and colon. The average count of IgA producing cells in colon was lower ($P < 0.05$) in piglets fed with FS on day 45 compared to control (Table 5).

Immunoglobulin quantities in serum

Comparing the effect of treatment on different immunoglobulin levels per sampling point, on day 45, FS treatment increased IgG quantity in blood compared to control ($P < 0.05$). No differences were observed for IgE levels on any of the sampling timepoints. Dietary treatment FS increased IgA level in blood compared to basal levels and to control on day 15 ($P < 0.05$). On day 15, FS

128 treatment tended ($P = 0.0660$) to increase IgM quantities in blood compared to control. On day
129 45, FS treatment increased ($P < 0.05$) IgM levels compared to control group (Table 6).

130 *Cytokine gene expression in tissue*

131 Several differences for cytokine expression were identified between dietary treatments. On day
132 15, a lower gene expression was observed for IFN- γ , IL-1 α and IL-10 ($P < 0.05$) and a tendency
133 ($P < 0.10$) for a lower expression of IL-1 β , IL-6 and TNF- α in the jejunum. There were no
134 differences on day 15 in ileum. In colon tissue, expression of IL-10 and IL-12 β tended ($P < 0.10$)
135 to be reduced in FS compared to control. In PBMC and on day 15, an increase in expression of
136 IFN- α , IL-6, IL-10, IL-12 α , IL-12 β and TGF- β was shown in FS compared to control.
137 Furthermore, tendencies for an increase ($P < 0.10$) were shown for IL-1 β and TNF- α in FS
138 treatment compared to control (Table 7).

139 On day 30, tendencies were observed for an increase ($P = 0.052$) in expression of IFN- α in the
140 jejunum, and for a reduction ($P = 0.050$) in TGF- β in FS treatment compared to control. In the
141 ileum, a tendency indicated a reduced expression of IL-10 ($P = 0.097$). In colon, a higher
142 expression of IL-10 ($P < 0.05$) and a tendency for an increase in IL-12 β in the FS treatment
143 compared to control were observed ($P = 0.085$). In PBMC, the FS treatment tended to increase
144 TGF- β expression ($P = 0.073$; Table 7).

145 On day 45, FS treatment reduced IL-1 α and IL-10 expression in the jejunum ($P < 0.05$),
146 increased ($P < 0.05$) IL-8 expression in the ileum, reduced ($P < 0.05$) IL-10 and tended ($P =$
147 0.070) to reduce IL-12 β expression in colon. Furthermore, in PBMC, IL-1 α expression was ($P <$
148 0.05) reduced by FS compared to control (Table 7).

Comparing gene expression of cytokines in jejunum, ileum, colon and PBMC over the three time points, it was observed that FS treatment reduced local tissue gene expressions of several cytokines, mainly on day 15 and 45, and increases gene expression in PBMC mainly on day 15 (Table 7).

Quantities of cytokines and lymphocytes in blood

Differences between control and FS fed pigs in the quantity of IL-10 in blood are presented in Table 8. Pigs fed FS show a decrease on day 15 compared to control ($P < 0.05$), and a tendency for a decrease on day 30 ($P = 0.080$; Table 8). No differences were observed in quantity of IFN- γ and the IFN- γ /IL-10 ratio between control and FS dietary treatments.

On day 15, quantities of NK cells were increased in FS compared to control ($P < 0.05$). On day 45, a decrease of B cells was observed in FS compared to control ($P < 0.05$). Lymphocytes Th tended to decrease on day 30 in pigs fed FS compared to control ($P = 0.081$). TCD3 quantity tended to decrease on day 15 in FS compared to control ($P = 0.0841$). On day 45, CD4-CD8- lymphocytes increased ($P < 0.05$), while CD4+CD8+ quantities decreased in FS compared to control ($P = 0.071$) (Table 9).

Discussion

Weaning-associated intestinal inflammation occurs in piglets (1,2). Determining nutritional strategies to prevent intestinal inflammation due to pathogenic enteric organisms can be an important pillar to maintain health status of animals. Present study provides evidence in immune modulation response induced by a blend of mannan-rich hydrolyzed copra meal and fermented rye with *A. subrufescens* in piglets post-weaning.

170 The primary focus of the current study was on piglet immunity and intestinal health while due to
171 design obvious reasons, the performance was secondary. Nonetheless, a tendency for improved
172 ADG was observed in piglets fed FS during the first 15 days which suggests a potential benefit of
173 FS effects on immunity and intestinal morphometry improving growth performance. There is little
174 research assessing such fungi origin feed additive (or similar components) effect on pig
175 performance. A report by the Danish National Committee for Pig Production described the effect
176 of 0.1% *A. subrufescens* extract added to weaner feed, and found no differences in diarrhea or
177 mortality, but an 11% increase in productivity in the supplemented group (25). In a Chinese study,
178 the effects were described of *A. subrufescens* improving the growth performance of weaned
179 piglets, where feed conversion rate was reduced (26). Next to performance improvement, the
180 researchers found that feeding soluble metabolites of *A. subrufescens* stimulated intestinal villous
181 development, which was also found in more recent research, where the supplementation of *A.*
182 *subrufescens* increased the villous height and the ratio of villous height/crypt depth of small
183 intestine in mice (27). These results imply that certain mycelial metabolites of *A. subrufescens*
184 might be important in enhancing villous development, which might consequently enhance the
185 absorption function of intestine. The use of FS in current study decreased villous height on day 15
186 in jejunum, while it increased the villous height in ileum on that same time point, and a similar
187 increase was seen on day 30 both in jejunum as in ileum. Peculiar about these results that the
188 reduction in villous height on day 15 in jejunum coincides with a tendency for improvement in
189 growth performance. In fact, the villous height, crypt depth and their ratio in the small intestine
190 are indexes to determine the capacity of intestinal absorption (28) and usually associated with an
191 improvement in growth performance (29). Nonetheless, there is also evidence that in germ-free
192 animals a slower turnover rate of intestinal epithelial cells resulted in longer villi (30). A slower

turnover is seen in neonatal pigs, with an epithelial cell turnover of 7 to 10 days compared to a 2- to 3-day turnover when pigs are 3 weeks of age (31). Therefore, further study may be required to clarify the mechanism of this improvement on FS on intestinal integrity and growth performance.

The quantity of IgA producing cells in jejunum, ileum and colon tissue was reduced in piglets fed FS. Reduced intestinal IgA producing cells in piglets fed FS coincided with elevated IgA and IgM levels in serum on day 15. Previous research performed in piglets has shown a significant decrease in piglet's plasma IgG concentrations immediately after weaning, which is in concurrence with the depletion of maternal immunity (2,4,32). In current study, piglets receiving FS had an increase in serum IgG quantity post-depletion at day 45. Previous in vivo results have shown the extract of the mushroom exhibited a significantly increased serum IgG level, T-cell population and phagocytic activity (33) in mice.

Looking at the adaptive immune response on a systemic level, analyses on lymphocyte quantities in blood demonstrated an increase in NK cells when feeding FS component. Interestingly, previous studies with the same mushroom fraction also enhanced the activity of NK cells in spleen (34,35). Yuminamochi et al. (36) demonstrated that powdered dried fruiting bodies and hemicellulase-digested component of *A. subrufescens* augmented NK cells activation through IL-12 mediated IFN- γ production. β -glucans and proteoglucans are known to be potent stimulators of macrophages (37–39), polymorph nuclear neutrophils (PMN) (40) and Natural Killer (NK) cells (41). In current study, besides an increase in amount of NK cells, lower amounts of T and B cells were observed in the treatment group at different timepoints. This can be of interest due to the previously reported downregulation of innate T helper pro-inflammatory pathways by β -1,4 mannobiose in a colitis pig model, induced by dextran sodium sulfate at 1.25 g/kg BW/day, which maintained intestinal permeability and histological morphology (42). The immunomodulatory properties of different β -

glucans have been demonstrated *in vitro* (17,43) and *in vivo* (44) to support intestinal health and microbiota balance in pigs (45,46).

Current study shows, that next to a reduction in IgA producing cells in the intestine, a decrease in intestinal cytokine production was seen in piglets fed FS, mainly in expression of IFN- γ , IL-1 α and IL-1 β , IL-6, IL-10 and TNF- α . Previous research has shown that challenging conditions can increase the gene expression of pro-inflammatory cytokines, such as the process of weaning or bacterial or infectious challenges. Studies provide evidence of cytokine regulation by weaning process with increased production of pro-inflammatory cytokines post-weaning (1,2,47). Production of intestinal mucosal IgA is controlled by cytokine-producing T cells within the GALT and by cytokines released from the mucosa. Within the GALT, the Th₁ cytokines, IFN- γ and TNF- β , downregulate IgA production, whereas the Th₂ cytokines, IL-4, IL-5, IL-6, and IL-10, upregulate IgA production (48,49). Several cytokines, such as TGF- β , IL-1 α , and IL-6 are constitutively expressed by the intestinal epithelium and may play a role in the normal influx of immune cells into the mucosa, in the growth of epithelial cells and in homeostasis (50). Other cytokines, such as IL-8, IL-1 β and TNF- α are also expressed by normal epithelial cells but are upregulated in response to microbial infection (51). The reduction in IgA secretory cells, combined with lower expression of cytokines in the intestinal mucosa could implicate a lower stimulated immune system, implicating a potential immunomodulating effect of FS.

This potential mode of action of fungal fermented products and their derivatives has been previously reported, where they are described to contain several compounds that may play a role in gastrointestinal health and pathogenic bacteria control (11). Promising examples are metabolites derived from edible mushrooms. Products from the *A. subrufescens* are reported to contain prophylactic and therapeutic properties, including antimicrobial and immunomodulatory (11,17).

239 *A. subrufescens*, also known as *A. blazei murill*, is an edible mushroom, which grows naturally in
240 Piedade, outside of São Paulo, Brazil. It contains high levels of biological response modulators,
241 such as proteoglycans (13,14) and β -glucans (15), which are a heterogeneous group of
242 polysaccharides present in cereal grains, fungal cell walls, seaweed, and algae (16). Microbial
243 enzymes produced by fungi during fermentation will degrade polysaccharides from feed material
244 into indigestible and bioactive oligosaccharides (12).

245 Many successful enteric organisms have developed strategies to resist displacement from the
246 epithelium via the development of anchoring adhesive fimbriae (pili) (52). Approaches to masking
247 these attachment sites from pathogenic bacteria include the feeding of competing carbohydrates
248 (oligosaccharides) that inhibit attachment of certain bacteria to the epithelium (53). Adhesins from
249 some pathogenic *Enterobacteriaceae* are known to show binding affinity to distinct indigestible
250 oligosaccharides. Wang et al., 2015 demonstrated such affinity for suitable oligosaccharides, i.e.,
251 d-mannose showed between 20% and 60% inhibition of bacterial adhesion (*E. coli*, *Vibrio*
252 *cholerae*, *Campylobacter jejuni*, and *Salmonella typhimurium*) to host glycans from HT-29 cells.
253 For example, addition of 2-5% D-mannose to broiler diets reduced the excretion and colonization
254 of *S. enterica* var. *typhimurium* (55), while the use of a mannan oligosaccharide has been reported
255 to reduce the concentration of caecal coliforms and *S. enterica* var. *typhimurium* and *S. dublin* in
256 chicks (52,56). A recent study demonstrated the effect of *in vitro* binding of FS, a blend of mannan-
257 rich hydrolyzed copra meal, containing levels of mannobiose, and a fungal fermented rye with *A.*
258 *subrufescens*, to *S. enterica* serovars *typhimurium* and *enteritidis*. *In vivo* results demonstrated that
259 feeding those components to nursery pigs reduced the peak and average *S. typhimurium* shedding
260 compared with control (57). Based on the recent data with FS and its components, in current study
261 the potential beneficial effect of FS against pathogenic bacteria by its binding capacity could have

lowered the amount of pathogenic bacteria in the lumen, or shifting microbiota reducing an immunological response initiated by the dendritic cells in the intestine. This can again result in a lower amount of IgA producing cells and a lower gene expression of cytokines such as IFN- γ , IL-1, IL-6, IL-10 and, IL-12 and TNF- α in the intestine. However, current study lacks data on the response of the intestinal microbiota to feeding component FS, therefore the exact mode of action of the lower intestinal inflammation response remains to be clarified.

Previous research done using the FS component in combination with organic acids indicated the potential in modifying the intestinal microbiota, with a higher abundance of *Lactobacillus spp* and a lower abundance of *Clostridium spp.* after a *S. typhimurium* challenge in post-weaning piglets (58). Furthermore, previous work in grower pigs showed that fed a dietary supplementation of *A. bisporus* (white button) mushrooms positively affected the composition of the fecal and proximal colon microbiota by promoting the abundance of *Ruminococcaceae* and *Lachnospiraceae* families (59). These families are known for degradation of complex plant material (cellulose and hemicellulose) in the mammalian gut and are considered as beneficial given their production of butyrate (60). The *A. bisporus* mushrooms fed to pigs did not affect growth rate, intestinal permeability or systemic and localized activation of mononuclear cells (59). However, an anti-inflammatory effect was observed in LPS-stimulated alveolar macrophages with a significant reduction in IL-1 β gene expression and cytokine production reflected in a lower activation of IL-1-signaling in pigs fed *A. bisporus* mushrooms.

Current study showed that reduced intestinal cytokine gene expression coincided with elevated cytokine gene expression in PBMC. This is in agreement with previous research where the use of *A. subrufescens* is shown to stimulate cytokine production in PBMC, such as interleukin-12 (IL-12) (34), or interferon- γ (IFN- γ) (36). Furthermore, the β -1,6-d-glucan fraction extracted from *A.*

subrufescens induces IFN- γ production and can partially reverse the production of IL-10 (61), the latter is in agreement with results of current study. *A. subrufescens* fraction had been shown to induce macrophages to secrete (TNF- α), (IL)-8 and nitric oxide (NO) in an in vitro test (62). Research in human medicine showed *A. subrufescens* extract to promote anti-inflammatory effects without side effects. Like in current study, pro-inflammatory cytokines (IL-1 β and IL-6) and chemokine (IL-8) were downregulated, and this was demonstrated ex vivo with heparinized blood of exposed Colitis Ulcerosa, Crohn's Disease and normal patients (63). These relatively common human diseases are characterized and a consequence of deficiency or enhanced activation of cytokine pathways, which promotes the breakdown of intestinal homeostasis (64,65). In pigs, more research is needed in this area. However, the reported post-weaning intestinal upregulation of pro-inflammatory cytokine production (1,2) demonstrates the potential of implementing nutritional strategies as shown in current study, with the aim to manipulate the immune system of pigs and reduce the immune response.

Conclusion

In piglets post-weaning, feeding component FS, a blend of mannan-rich hydrolyzed copra meal and fermented rye with *A. subrufescens*, stimulates an immune modulation effect most evident at 15 days post-weaning. The effect includes a reduction of local intestinal inflammatory response, with emphasis in jejunum, accompanied with an increase in systemic (PBMCs) cytokine gene expression. Additionally, increased villous height in jejunum and ileum on day 30, while it was reduced in jejunum on day 15.

Methods

Animals, housing, and experimental design

Current study was performed at the Veterinary Teaching Farm of the University of Murcia (Spain), using 71 piglets (Large White) weaned at 22 ± 3 days (d) of age with an average initial body weight (BW) of 5.53 ± 1.19 kg and about 1:1 male:female ratio. After weaning, all pigs (segregated by gender and weight) were randomly allotted to two treatments with 8 pens per treatment and 9 animals per pen (pen size: 0.61×1.22 m). Pigs were housed in an environmentally controlled unit with natural light throughout the experiment. The pens were full-slatted (plastic slat) and contained one nipple drinker *ad libitum* and one feeder with 4 spaces. On day 4 post-weaning piglets were routinely vaccinated against PCV2 (Porcilis PCV-2, MSD) and no systematic medications were included in feed or water.

Diets and additives

A two-phase experimental diet was used, produced at a local feedmill (Pigalomar; Spain), without additional additives or medication but the dietary treatments reported (Table 1). All diets were pelleted (4 mm) and were formulated to meet the current estimates for nutrient growth requirements for nursery pigs (NRC, 2012). Diets did not contain spray-dried plasma, antibiotics, and pharmaceutical levels of zinc oxide. The dietary treatments consisted of a control diet and a treatment diet, being the control diet + 0.2% Fysal Solute (FS), a feed additive consisting of a blend of mannan-rich hydrolyzed copra meal and fermented rye with *A. subrufescens*. The feed additive used in the present experiment was provided by Trouw Nutrition, The Netherlands.

Growth performance and sample collection

Individual body weight of piglets was recorded at day 0 (weaning), day 15, day 30 and day 45, and average daily gain was calculated. At weaning (weaning + 18 hours), 10 control animals were sacrificed to obtain blood from the vena jugular and intestinal tissue samples as a basal

treatment. Animals were euthanized by lethal intravenous injection with an overdose of tiobarbital IV (50 mg/kg BW, Tiobarbital Braun Medical S.A., Barcelona, Spain). Subsequently, sacrifice was done at day 15 (control n=11, FS n=10), day 30 (control n=6, FS n=12) and day 45 (control n=9, FS n=13) to obtain blood and intestinal tissue samples. Tissue samples were obtained from the jejunum (middle section), ileum (5 cm adjacent to ileocecal valve), and colon (apex section of spiral) by three centimeter long gut sections, opened along the mesenterial insertion and 2 cm cut from their middle section. After being gently washed in water, each sample was divided in two subsamples, one (20 mg) was preserved in RNAlater (Life Technologies, USA) and one was fixed in 10% buffered formaldehyde and embedded in paraffin-wax for histomorphometrical and immunohistochemical studies. Blood samples were collected into ethylenediaminetetraacetic (EDTA) tubes (Vacutainer, Becton Dickinson, UK). Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll Histopaque gradient and preserved in RNAlater (Life Technologies, USA) at -80°C, after 24 hours of refrigeration at 8°C. Serum and plasma were isolated from whole blood by centrifugation (251 rcf, 10 min at room temperature) and preserved at -80°C up to analysis.

Sample analysis

Total serum immunoglobulins IgA, IgE, IgG and IgM were quantified in an amount of 50 µL serum sample by Enzyme Linked Immuno Sorbent Assay (ELISA) (Elabscience, USA) per manufacturer instructions. For establishment of the immunotype Th1/Th2, the cytokines IL-10 and IFN- γ have been quantified in 50 µL of serum sample by means of ELISA (Invitrogen, USA) as per manufacturer instructions.

Lymphocytes B, T and NK

Flow cytometry was used to analyse subpopulations of different types of lymphocytes: B (phenotype CD21+), NK (phenotype CD3-16+56+) and T phenotypes CD3+, T helper CD3+CD4+, T cytotoxic CD3+CD8+, CD4+CD8+, CD4-CD8-. A sample of 50 µL of peripheral blood per tube was mixed with antibodies according to manufacturer. Commercial antibodies were used separately for each tube, using mouse anti-pig CD56:RPE (BioLegend, CA, USA), mouse anti-pig CD16:RPE and mouse anti-pig CD45:FITC (Bio-RAD, CA, USA), PerCP-Cy5.5 mouse anti-pig CD4a, mouse anti-pig CD8a, PE-Cy7 mouse anti-pig CD3e, and PE-Cy5 mouse anti-human CD21 (BD Pharmingen, Becton, Dickinson and Company, NJ, USA). Post incubation, the sample was lysed with a commercial lyse (FACS LYSING, Becton, Dickinson and Company, NJ, USA), after which sample was analysed in the FC500 cytometer (Beckman Coulter, IN, USA) by using a single 488 nm (blue) laser and obtaining analysis matrices for each analyte.

Immunohistochemistry for quantity of IgA producing cells

In jejunum, ileum and colon tissue IgA secretory cells were detected using the avidin- biotine- peroxidase complex technique. From the samples embedded in paraffin-wax 5µm thick slides were obtained, dewaxed and dehydrated with graded ethanol and the endogenous peroxidase activity was quenched in 3% H₂O₂ in methanol for 30 min. Samples were treated with 10% pronase in TBS (Sigma-Aldrich, USA) for antigen retrieval (12 min). After pretreatment, samples were rinsed three times in TBS for 5 min each and incubated for 30 min with 100 µL of blocking solution per slide at room temperature in a humid chamber, before incubation for 1h at 37 °C with the primary antibody goat anti-pig IgA (Bethyl, USA) diluted 1:3000 in TBS. The secondary antibody biotin conjugate rabbit anti-goat (Dako, USA) diluted 1:300 in TBS was incubated for 30 min at room temperature. The Vectastain® Elite ABC kit (Vector, USA) was applied for 1h at room temperature. Positive labeling was detected using 3,3'-diaminobenzidine

374 tetrahydrochloride (Dako, USA). Sections were counterstained with Mayer's haematoxylin,
375 dehydrated and mounted. By using a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen,
376 Germany) with a Spot Insight camera and the Spot Advanced software (Spot Imaging Solution,
377 Michigan, USA) the number of IgA secretory cells in the intestinal lamina propria was counted.
378 Immunolabeled cells were counted in 10 non-overlapping consecutive high magnification fields
379 of 25.000 μm^2 .

380 Cytokine gene expression

381 Gene expression for cytokines IL-1 β , IL-6, IL-8, IL-10, IL-12p35 (IL-12 α), IL-12p40 (IL-12 β),
382 TNF- α , IFN- α , IFN- γ and TGF- β was analysed using relative quantification, with primers
383 previously described in literature (see table 2). Total RNA was isolated from tissue and PBMCs
384 samples by means of Micro RNeasy kit (Qiagen, USA) and DNase was synthesized using the
385 Geneamp RNA PCR Core Kit (Life Technology, USA). The PCRs were performed using a 7300
386 ABI thermocycler (Life Technologies, USA) and the GoTaq® qPCR Master Mix (Promega,
387 USA) with SYBR-Green chemistry. The specificity of the reaction was assessed by analyzing the
388 melting curve. The samples were normalized using the average Ct for glyceraldehyde-3-
389 phosphatedehydrogenase (GAPDH), cyclophilin and β -Actin. The expression for each sample
390 was calculated (66), correcting to the PCRs efficiency, calculated by serial decimal dilutions and
391 using the slope offered by the thermacycler software, and used as control group the animals
392 sampled at day 0. The efficiency of q-PCRs was between 91% and 105%. Data were expressed
393 as fold change, normalized to the lowest value (which was assigned a value 1).

394 Intestinal histomorphometry

Five photomicrographs were taken with a Zeiss Axiocam 503 color (Carl Zeiss, Oberkochen, Germany) coupled to a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany) with 10x magnification, from each section of jejunum and ileum. Both the height of the villous (tip to villous-crypt junction) and depth of the crypt (from villous-crypt junction to the base of villous) were analysed with the ZEISS Efficient Navigation software (Carl Zeiss, Oberkochen, Germany), according to manufacturer instruction. Randomly selected well-oriented intact villi and crypts (n=10) were measured per piglet, per timepoint for jejunal and ileal tissue. The mean villous height and crypt depth of each intestinal tissue was calculated and by dividing villous height by crypt depth the villous height/crypt depth ratio was calculated. All morphometric measurements were performed by the same blinded to treatments researcher.

Statistical analysis

Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute; Cary, USA). The cytokine gene expression data was transformed as Log2 of the value for normalization. The model included the fixed effects of treatment. Animal was the experimental unit. Significantly different means were separated using Tukey adjust. Statistical significance and tendency were considered at $P \leq 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

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625

626   **Declarations**

627   **Ethics approval and consent to participate**

628   All the experimental procedures described in this research were reviewed and approved by the
629   Bioethical Committee of the University of Murcia and applied under project license permit CEEA-
630   OH 465/2018.

631   **Consent of publication**

632   Not applicable

633   **Availability of data and materials**

634   The datasets supporting the conclusions of this article are included within the article (and its
635   additional files.

636   **Competing interests**

637   The authors have declared that no competing interest exists.

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641   **Authors' contributions**

642   NG analyzed and interpreted the data and prepared first draft of the manuscript and was the
643   major contributor in writing. GR, FF and CCG performed the DNA extraction, qPCR analysis and
644   the histological examination of jejunum. LF contributed to reviewing the manuscript.

645 Table 1. Composition of the experimental diets

Item	Phase 1 day 0-14	Phase 2 Day 15-45
Ingredients, %		
Barley	29.98	25.00
Wheat	24.00	26.89
Corn	17.17	19.50
Soybean Meal 47 crude protein	6.00	16.83
Ca carbonate	0.45	0.61
Monocalcium phosphate	0.75	0.78
Soybean oil	3.50	3.67
Intestinal swine mucose hydrolyzate	2.50	0.00
Milkpowder	5.00	0.00
Fysal MP ¹	0.30	0.30
Salt	0.30	0.44
L-Valine (96.5%)	0.050	0.025
DL-Methionine (99%)	0.175	0.175
L-Lysine HCl (98%)	0.542	0.525
L-Threonine (98%)	0.258	0.250
L-Tryptophan (98%)	0.033	0.008
Protein concentrate ²	6.00	2.00
TN Trouwmix 30 premix ³	3.00	3.00
Calculated content ⁴ , %		
Dry matter	89.60	89.17
Net energy, kcal	2,559	2,549
SID lysine	1.15	1.17
SID methionine	0.405	0.406
SID methionine + cysteine	0.686	0.705
SID tryptophan	0.241	0.230
SID threonine	0.750	0.761
Crude protein	16.67	17.72
Crude fiber	2.98	3.14
Ash	4.72	4.74
Neutral detergent fiber	11.3	11.7
Sodium	0.24	0.22
Chloride	0.45	0.44
Calcium	0.55	0.59
Phosphorus	0.55	0.55
Copper, mg/kg (as SO ₄)	150	150
Manganese, mg/kg (as MnO)	50	50
Zinc, mg/kg (as ZnO)	105	105

646 ¹blend of free and buffered organic acids,

647 ²gluten meal, extruded soybean meal, potato protein

648 ³Vitamin and mineral premix provided the following per kilogram of diet: vitamin A, 15,000 IU;
649 vitamin D, 2000 IU; vitamin E, 100 IU; 30 µg of vitamin B12; vitamin K, 2 mg; D-
650 pantothenic acid 15 mg as calcium pantothenate; 30 mg of nicotinic acid; choline, 150 mg as
651 betaine hydrochloride; Mn, 50 mg as manganese oxide; Zn, 105 mg as zinc oxide; Fe, 100 mg as
652 iron sulphate; Cu, 120 mg as copper sulphate; I, 1.5 mg as potassium iodide; Se, 0.42 mg as
653 sodium selenite; 6-phytase 1500 FTU.

654 ⁴Nutrient levels are calculated. SID means standardized ileal digestible.

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657 Table 2. Primers of the cytokines IL-1 β , IL-6, IL-8, IL-10, IL-12p35 (IL-12 α), IL-12p40 (IL-
658 12 β), TNF- α , IFN- α , IFN- γ and TGF- β and primers of primers of glyceraldehyde-3-
659 phosphatedehydrogenase (GAPDH), cyclophilin and β -actin

	Primer forward (5' $\rightarrow$ 3')	Primer reverse (5' $\rightarrow$ 3')	References
IFN- α	5'-CCCCTGTGCCTGGGAGAT-3'	5'-AGGTTTCTGGAGGAAGAGAAGGA-3'	(67)
IFN- γ	5'-TGGTAGCTCTGGGAAACTGAA TG-3'	5'-GGCTTTGCGCTGGATCTG-3'	(68)
TNF- α	5'-ACTCGGAACCTCATGGACAG-3'	5'-AGGGGTGAGTCAGTGTGACC-3'	(69)
IL-12p35	5'-AGTTCCAGGCCATGAATGCA-3'	5'-TGGCACAGTCTCACTGTTGA-3'	(67)
IL-12p40	5'-TTTCAGACCCGACGAACTCT-3'	5'-CATTGGGGTACCAGTCCAAC-3'	(70)
IL-10	5'-TGAGAACAGCTGCATCCACTT C-3'	5'-TCTGGTCCTTCGTTTGAAAGAAA-3'	(68)
TGF- β	5'-CACGTGGAGCTATAACCAGAA-3'	5'-TCCGGTGACATCAAAGGACA-3'	(67)
IL-8	5'-GCTCTCTGTGAGGCTGCAG TTC-3'	5'-AAGGTGTGGAATGCGTATTTAT GC-3'	(71)
IL-1 α	5'-GTGCTCAAAACGAAGACGAA CC-3'	5'-CATATTGCCATGCTTTTCCCAGA A-3'	(72)
IL-1 β	5'-AACGTGCAGTCTATGGAGT-3'	5'-GAACACCACTTCTCTCTTCA-3'	(73)
IL-6	5'-CTGGCAGAAAACAACCTGAA CC-3'	5'-TGATTCTCATCAAGCAGGTCTCC-3'	(73)
β -actin	5'-CTACGTCGCCCTGGACTTC-3'	5'-GATGCCGCAGGATTCCAT-3'	(74)
Cyclophilin	5'-TGCTTTCACAGAATAATTCCA GGATTTA-3'	5'-GACTTGCCACCAGTGCCATTA-3'	(75)

GAP 5'- 5'- (75)
DH ACATGGCCTCCAAGGAGTAAG GATCGAGTTGGGGCTGTGACT-3'
A-3'

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Table 3 - Effect of dietary treatment¹ on performance of weaned piglets at different time points

	Day	Control	FS ¹	SEM	P-value
BW, kg	0	5.59	5.97	0.288	0.705
	15	8.84	10.4	0.972	0.111
	30	14.5	15.6	1.41	0.467
	45	17.9	20.7	2.81	0.318
ADG, kg/d	0-15	0.199 ^y	0.297 ^x	0.055	0.079
	0-30	0.288	0.320	0.038	0.402
	0-45	0.305	0.330	0.047	0.605
	30-45	0.358	0.408	0.059	0.409

SEM = standard error of the mean. BW = body weight; ADG = average daily gain. ^{x-y} Values

within a row with different superscripts trend at $P < 0.10$.

¹Dietary treatment = additional Fysal Solute (FS) at 2 kg/ton

Table 4 - Effect of dietary treatment¹ on intestinal histomorphometry measurements in tissue of piglets at different time points

Day	Tissue	day	Treatment		SEM	P-value
			Control	FS ¹		
15	Jejunum	Villous length	492 ^a	438 ^b	16.8	0.035
		Crypt depth	292	317	14.9	0.264
		V/C ratio	1.36	1.13	0.178	0.358
	Ileum	Villous length	414 ^x	452 ^y	15.3	0.093
		Crypt depth	258	297	17.0	0.116
		V/C ratio	1.50	1.57	0.104	0.657
30	Jejunum	Villous length	447 ^a	519 ^b	13.5	0.002
		Crypt depth	319	363	20.1	0.168
		V/C ratio	1.02	0.836	0.162	0.431
	Ileum	Villous length	403 ^x	459 ^y	21.3	0.093
		Crypt depth	354	320	23.9	0.356
		V/C ratio	1.18 ^a	1.46 ^b	0.084	0.035
45	Jejunum	Villous length	516	515	24.1	0.969
		Crypt depth	317	354	21.0	0.227
		V/C ratio	0.930	0.814	0.074	0.287
	Ileum	Villous length	452	497	21.7	0.234
		Crypt depth	324	305	16.8	0.428
		V/C ratio	1.49	1.55	0.100	0.717

SEM = standard error of the mean. ^{a-c} Different superscripts within a row indicate a significant difference ($P < 0.05$)

¹Dietary treatment = additional Fysal Solute (FS) at 2 kg/ton

Table 5 - Effects of dietary treatment¹ on Immunoglobulin A producing cells in tissue of piglets at different time points

Tissue	day	Treatment		SEM	P-value
		Control	FS ¹		
Jejunum	15	13.6 ^a	7.3 ^b	1.03	<.00
	30	23.0 ^a	17.3 ^b	1.32	0.009
	45	26.0	23.5	2.86	0.544
Ileum	15	11.8 ^a	6.62 ^b	0.873	0.001
	30	20.2 ^a	13.4 ^b	1.229	0.002
	45	18.7	16.4	2.05	0.429
Colon	15	11.3	8.98	1.032	0.130
	30	19.7 ^a	15.6 ^b	1.437	0.001
	45	23.0 ^a	17.9 ^b	1.273	0.012

SEM = standard error of the mean. ^{a-b} Different superscripts within a row indicate a significant difference ($P < 0.05$)

¹Dietary treatment = additional Fysal Solute (FS) at 2 kg/ton

Table 6 – Effects of dietary treatment¹ on immunoglobulin quantities in serum of piglets on different time points

Immunoglobulin	Day	Treatments		SEM	P-value
		Control	FS ¹		
IgG (mg/mL)	15	5.89	6.45	0.707	0.595
	30	6.74	7.65	0.492	0.243
	45	7.67 ^a	9.94 ^b	0.619	0.047
IgE (ng/mL)	15	22.4	25.0	1.57	0.277
	30	22.0	31.7	3.72	0.110
	45	25.6	21.0	3.84	0.443
IgA (ug/mL)	15	72.4 ^a	101 ^b	7.26	0.026
	30	97.1	107	7.57	0.371
	45	108	106	31.6	0.964
IgM (ug/mL)	15	62.3 ^x	119 ^y	18.2	0.066
	30	114	153	14.9	0.110
	45	111 ^a	151 ^b	8.82	0.019

SEM = standard error of the mean. ^{a-c} Different superscripts within a row indicate a significant difference ($P < 0.05$), ^{x-y} Different superscripts within a row indicate a trend difference ($P < 0.10$)

¹Dietary treatment = additional Fysal Solute (FS) at 2 kg/ton

Table 7 - Effects of dietary treatment¹ on cytokine expression in tissue of piglets at different timepoints

Day	Tissue	IFN- α	IFN- γ	IL-1 α	IL-1 β	IL-6	IL-8	IL-10	IL-12a	IL-12 β	TNF- α	TFG- β
d15	Jejunum		<*	<*	<^	<^		<*			<^	
	Ileum											
	Colon							<^		<^		
	PBMC	>*		>*	>^	>*	>^	>*	>^	>*	>^	>*
d30	Jejunum	>^										<^
	Ileum							<^		<^		
	Colon							>*		>^		
	PBMC											>^
d45	Jejunum		<*	<*				<*				
	Ileum						>*					
	Colon		<^					<*		<^		
	PBMC				<*							

(*) Significant ($P < 0.05$) and (^) tendency ($P < 0.1$) upregulation (>) or downregulation (<) of cytokine mRNA levels of FS compared to control.

¹Dietary treatment = additional Fysal Solute (FS) at 2 kg/ton

Table 8 - Effects of dietary treatment¹ on IFN- γ and IL-10 (pg/mL) and the IFN- γ /IL-10 ratio in blood of piglets at different timepoints

Day	Parameter	Control	FS ¹	SEM	P-value
d 15	IFN- γ	7.55	12.9	3.76	0.189
	IL-10	11.9 ^a	3.53 ^b	3.13	0.028
	Ratio IFN- γ /IL-10	0.823	8.35	4.37	0.123
d 30	IFN- γ	4.48	3.54	0.999	0.377
	IL-10	51.3 ^x	17.6 ^y	16.5	0.080
	Ratio IFN- γ /IL-10	0.207	0.789	0.841	0.511
d 45	IFN- γ	3.26	3.99	1.526	0.648
	IL-10	6.46	6.24	2.587	0.934
	Ratio IFN- γ /IL-10	0.479	0.801	0.373	0.416

SEM = standard error of the mean. ^{a-b} Different superscripts within a row indicate a significant difference ($P < 0.05$), ^{x-y} different superscripts within a row indicate a tendency difference ($P < 0.1$)

¹Dietary treatment = additional Fysal Solute (FS) at 2 kg/ton

769 Table 9 - Effects of dietary treatment¹ on lymphocytes quantification in blood of piglets at
770 different timepoints

Lymphocyte	Day	Control	FS¹	SEM	P-value
NK	15	27.3 ^a	32.2 ^b	1.52	0.035
	30	23.3	29.3	3.73	0.278
	45	36.8	32.4	1.93	0.127
B	15	11.3	10.3	1.96	0.740
	30	12.3	9.66	1.24	0.142
	45	10.3 ^a	7.9 ^b	0.647	0.017
Th	15	25.3	23.7	1.86	0.555
	30	33.8 ^x	27.4 ^y	2.34	0.081
	45	27.1	27.4	1.19	0.849
Tc	15	17.9	18.4	2.02	0.879
	30	20.9	20.6	2.05	0.944
	45	15.7	15.7	1.78	0.999
TCD3	15	62.3 ^x	53.1 ^y	3.52	0.084
	30	55.7	49.6	3.14	0.192
	45	50.5	50.5	2.50	0.991
CD4-CD8-	15	7.06	7.80	1.52	0.733
	30	12.4	10.2	1.09	0.179
	45	8.2 ^a	10.8 ^b	0.800	0.033
CD4+CD8+	15	49.0	44.8	2.74	0.298
	30	36.9	38.5	3.17	0.740
	45	39.2 ^x	33.9 ^y	1.94	0.071

771 SEM = standard error of the mean. ^{a-b} Different superscripts within a row indicate a significant
772 difference ($P < 0.05$), ^{x-y} different superscripts within a row indicate a tendency difference ($P <$
773 0.1)

774 ¹Dietary treatment = additional Fysal Solute (FS) at 2 kg/ton

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