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^{1}H-NMR metabolomics response to a realistic diet contamination with the mycotoxin deoxynivalenol: Effect of probiotics supplementation.

Imourana Alassane-Kpembi[1,2]*, Cecile Canlet[1], Marie Tremblay-Franco[1], Fabien Jourdan[1], Maxime Chalzaviel[3], Philippe Pinton[1], Anne Marie Cossalter[1], Caroline Achard[4], Mathieu Castex[4], Sylvie Combes[5], Ana Paula L. Bracarense[6], and Isabelle P. Oswald[1]*

[1]Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-Purpan, UPS, Toulouse, France.
[2]Université d'Abomey-Calavi, Ecole Polytechnique d'Abomey-Calavi, Bénin
[3]MedDay Pharmaceuticals, Paris, France.
[4]Lallemand SAS, 19 rue des Briquetiers, BP 59, 31702 Blagnac cedex, France.
[5]GenPhySE, Université de Toulouse, INRA, ENVT, Castanet Tolosan, France
[6]Universidade Estadual de Londrina, Lab. Patologia Animal, Londrina, Brazil
* Correspondence: iakpembi@gmail.com Tel: +33625234062; isabelle.oswald@inra.fr

Running title: Metabolomic response of DON-challenged piglets

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Keywords: Mycotoxin; *Saccharomyces cerevisiae* boulardii; pig; metabolomics; histology; intestine.

Abstract

Low-level contamination of food and feed by mycotoxin deoxynivalenol (DON) is unavoidable.
We investigated the effects of subclinical challenge with DON, and dietary supplementation with probiotic yeast *Saccharomyces cerevisiae* boulardii *I1079* as preventive strategy.
Thirty-six piglets were randomly assigned to four different diets: control diet, diet contaminated with DON (3 mg/kg), diet supplemented with yeast ($4x10^9$ CFU/kg), or DON-contaminated diet supplemented with yeast, for four weeks. Plasma samples were collected for biochemistry, and tissue samples for histology. ^{1}H-NMR untargeted metabolomics of plasma and liver were also explored.
DON induced no significant modification of biochemistry parameters. However, higher lesional scores were observed and metabolomics highlighted alteration of the metabolism of amino acids and 2-oxocarboxylic acids. Administration of yeast impacted aminoacyl-tRNA synthesis and metabolism of amino acids and glycerophospholipids. Yeast supplementation to DON-exposed piglets prevented histological alterations, while partial least square discriminant analysis underlined similarity of their plasma metabolic profile to control group. In contrast to plasma, the effect on liver metabolome remained marginal, indicating that the toxicity of the mycotoxin was not abolished.
These data indicate that ^{1}H-NMR metabolomics profile is a good biomarker for subclinical exposure to DON, and supplementation with *S. cerevisiae* boulardii increases piglet resilience to this mycotoxin.

Keywords: Mycotoxin; *Saccharomyces cerevisiae* boulardii; pig; metabolomics; histology; intestine.

1. Introduction

Deoxynivalenol (DON) belongs to the trichothecene group of mycotoxins; these are closely related sesquiterpenoid compounds with a 12,13-epoxy ring and a variable number of hydroxyl, acetoxy or other substituents [1]. Owing to the large-scale infestation of wheat, barley and corn by the *Fusarium* fungi both in the field and during storage, DON is an important food and feed safety issue in Europe and North America [2, 3]. At the molecular level, DON induces ribotoxic stress which disrupts normal cell function by impairing macromolecule synthesis and activating critical cellular kinases involved in signal transduction related to proliferation, differentiation, and apoptosis [1]. The pathophysiological effects associated with DON include altered neuroendocrine signalling, pro-inflammatory gene induction, disruption of the growth hormone axis, and altered gut integrity [4]. In animal studies, chronic exposure has been shown to result in impaired weight gain, anorexia, haematotoxicity and immune dysregulation, although in farm animals there are marked differences in DON toxicity between species , and pigs are the most sensitive [5].

The prevalence of acute or clinical toxicosis cases linked to DON, and to other mycotoxins has been drastically reduced in the framework of the food and feed regulations implemented in several countries [6, 7]. Nonetheless, unavoidable low-noise mycotoxin contamination, particularly of feed, means these compounds continues to be important players in the global health status of herds, and makes it necessary to resort to other strategies including detoxification or supplementation with probiotics [8-10].

Probiotic *Saccharomyces cerevisiae* strains have demonstrated their potential to tackle a variety of factors threatening pig intestinal health [11-13]. Trophic effects resulting in faster tissue reconstitution and anti-inflammatory effects on intestinal mucosa have revealed, particularly for *Saccharomyces cerevisiae* boulardii [14-16]. Concerning injury to intestinal tissue caused by a mycotoxin, we previously showed that the yeast significantly reduced the global impact of DON on the intestinal transcriptome, and reversed prototypical inflammation signalling pathways including NF-κB and p38 MAPK [17]. In the case of the methotrexate or D-galactosamine or tetrachloride induced toxicities in rodents, the administration of *S. cerevisiae* boulardii to rodents not only promoted recovery of the intestinal injury, but also alleviated acute liver lesions, suggesting that the protective effect of the yeast may extend to internal organs [18-20].

By focusing on the small molecular weight molecules or metabolites that are present in biological samples, metabolomics profiling can identify perturbations in metabolism under different conditions. The metabolome responds to nutrients, stress or disease long before classical biomarkers, making it an attractive approach for multiple fields, with metabolite alterations now implicated in the development of a number of diseases [21]. In addition, metabolomics can be used to monitor the outcome of treatment strategies including dietary interventions, by observing whether the metabolic phenotypes of treated shifts in the cluster of healthy subjects [22].

The aim of the present study was to provide a metabolic fingerprinting of a subacute exposure to DON at a concentration that is likely to occur in food or feed, and to evaluate the potential of the supplementation with *S. cerevisiae* boulardii I1079 strain to counteract the mycotoxin effects.

2. Material and methods

2.1. Animals, diets and tissue/blood sampling

Thirty-six crossbred castrated male piglets weaned at 28 days of age were used in the present study. All animal experimentation procedures were carried out in accordance with the European Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU), and validated by the Ethics Committee for Animal Experiments Toxcomethique APAFIS#8280-2016122010097752v3. Four of the authors (IAK, PP, SC and IPO) have an official agreement from the French Veterinary Services for animal experimentation.

The piglets were acclimatized for one week in the animal facility of the INRA ToxAlim Laboratory (Toulouse, France) before the experiment began. Animals had *ad libitum* access to water and feed throughout the experiment.

The animals received one of four dietary treatments for 28 days: basal diet (CTRL); basal diet supplemented with *S. cerevisiae* boulardii *CNCM I-1079* ($4x10^9$ CFU/kg feed), DON-contaminated basal diet (2.82 mg DON/kg feed,) supplemented or not with *S. cerevisiae* boulardii *CNCM I-1079*.

The mycotoxin content of the feed was analysed for (Labocea, Ploufragan, France). Fumonisin B_1 and B_2 were naturally present in the cereals used, resulting in concentrations between 0.01 and 0.04 mg/Kg feed. All other mycotoxins, including zearalenone and its metabolites, aflatoxins, T-2 toxin, HT-2 toxin, and ochratoxin A, were below the limits of detection. Purified DON obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France) was used to contaminate the diet. *S. cerevisiae* boulardii *CNCM I-1079* was from Lallemand Animal Nutrition (Blagnac, France). The basal diet formulation and nutrient contents are listed in Table 1.

At weekly intervals, blood samples were aseptically collected from the left jugular vein in tubes containing sodium heparin (Vacutainer®, Becton-Dickinson, USA). Plasma samples were obtained after centrifugation of heparinized blood and stored at −20°C for later analysis. At the end of exposure period, the piglets were fasted overnight before being subjected to electrical stunning and euthanized by exsanguination. Samples were collected from the intestine (mid-jejunum), liver and kidney and snap frozen for metabolomics analysis or fixed in 10 % buffered formalin solution for histological analysis.

2.2. Plasma biochemistry

Concentrations of albumin, cholesterol, creatinine, glucose, lactate, magnesium, phosphorous, total proteins, triglycerides, urea, and activity of lactate deshydrogenase, aspartate aminotransferase, alkaline phosphatase, alanine aminotransferase in the plasma were measured with a Pentra 400 Clinical Chemistry benchtop analyzer (Horiba, Les Ulis, France) at GenoToul-Anexplo platform (Toulouse, France).

2.4. Assessment of tissue lesions and villous morphometry

The pieces of tissue were dehydrated in graded alcohol and embedded in paraffin wax. Sections (3 µm) were stained with hematoxylin-eosin (HE, Sigma) for histopathological evaluation. Microscopic observations of the jejunum, liver and kidney were quantified as previously described [23, 24] with minor modifications (please see Supplemental Table 1 and Supplemental methods).

Morphometry was assessed in the jejunum by measuring the villi height on 30 randomly selected villi using a MOTIC Image Plus 2.0 ML® image analysis system. To evaluate lesions and

morphometry in these tissues, the slides were observed in a blind way by the same pathologist, irrespective of the experimental groups.

2.5. Statistical analysis of zootechnical, biochemistry and histological data

For the blood biochemistry parameters, a two-way ANOVA, using diet and sampling day as factors with a Tukey multiple comparisons of means in post-hoc, was performed to analyse the differences between the experimental groups. Data that did not follow a normal distribution were log-transformed prior to the statistical analysis. The weight gain and histological data analysis was done by one way-ANOVA with a Bonferroni test as post-hoc. Data that did not follow a normal distribution were analysed by Kruskal–Wallis non-parametric test followed by the Mann-Whitney U test. $P < 0.05$ was considered significant.

2.6. NMR-based metabolomics analyses

2.6.1. ^{1}H NMR analyses

^{1}H NMR spectra for the metabolic fingerprinting of plasma and liver samples were obtained at 300 K on a Bruker AVANCE III HD 600 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz for ^{1}H resonance frequency using an inverse detection 5 mm ^{1}H-^{13}C-^{15}N-^{31}P cryoprobe attached to a cryoplatform (the preamplifier cooling unit). Plasma and liver sample preparation, and analysis were performed as previously described [25]. To confirm the chemical structure of metabolites of interest, two-dimensional (2D) ^{1}H-^{1}H COSY (correlation spectroscopy) and 2D ^{1}H-^{13}C-HSQC (heteronuclear single quantum coherence spectroscopy) NMR experiments were performed on selected samples. For more details on plasma and liver sample preparation, analysis and data reduction, please see Supplemental methods.

2.6.2. Multivariate and univariate statistical analyses.

Multivariate analyses were used to study the effect of the treatment (control or *S. cerevisiae* boulardii or DON or DON+ *S. cerevisiae* boulardii) on the metabolome. We first performed principal component analysis (PCA) to reveal intrinsic treatment-related clusters and detect eventual outliers. Partial least square discriminant analysis (PLS-DA) was then used to model the relationship between group and spectral data. Before analysis, we used orthogonal signal correction filtering [26] to remove variation not linked to the treatment (physiological, experimental, or instrumental variation). Filtered data were mean centered and scaled (Pareto scaling). The number of components in the PLS models was chosen by 7-fold cross validation. The R^2 parameter represents the explained variance. The predictive performance of the model was evaluated using the Q^2 parameter (predictive capacity), calculated by cross-validation. AUROC (Area Under the Receiver Operating Curve) and misclassification rate (number of observations classified in the wrong group) were used to assess precision of PLS-DA models. The Q^2 intercept values of a permutation test with 200 permutations was used to assess robustness of PLS-DA models. A robust model has a Q^2 intercept value < 0 [27]. Variable importance in the projection (VIP) was used as global measure of the influence of each variable on the PLS components to derive a subset of the most important metabolites for the separation of the experimental groups. The Kruskal–Wallis test followed by the Mann-Whitney U test were then used to determine which metabolites differed significantly between groups. False Discovery Rate (FDR) correction was used to take into account multiple testing. SIMCA-P software (14; Umetrics AB, Umea, Sweden) was used for multivariate analyses. Univariate analyses were performed with R software (www.r-project.org).

2.6.3. Extraction of modulated metabolic networks

Genome scale metabolic network (GSMN) analysis was performed as previously described [28]. We used the *Sus scrofa* GSMN which was imported in MetExplore web server from KEGG database (MetExplore network 4105, downloaded from KEGG database May 25th 2016) [29, 30]. The network contains 1807 metabolic reactions and 1520 metabolites.

We performed metabolic sub-network extraction from the discriminant metabolites identified in the statistical analyses [31].

All computational and visualization tasks were performed on the MetExplore web server (version 2.18.19)[29].

3. Results

3.1. Effects of DON and *S. cerevisiae* boulardii supplementation on piglet weight gain and biochemical parameters

The body weight gain and plasma biochemistry parameters recorded for the four experimental groups on a weekly basis are presented in Figure 1 and Table 2.During the experiment, no significant difference in piglet weight gain was observed between the different experimental groups (Fig 1). Except for an increase of alanine aminotransferase, a classical marker of hepatic dysfunction, that was not confirmed by the other tested transaminase (AST) in the DON group, no significant alteration of plasma biochemical profile in any of the groups was reported at the end of the experiment (Table 2).

3.2. Supplementation of *S. cerevisiae* boulardii protects piglet tissue from the DON-induced subclinical lesions

Figure 2 presents tissue alterations observed on jejunum, liver and kidney in the four experimental groups.

In the intestine, significant histological alterations were observed over the four week dietary exposure to DON (Fig 2A). The main histological lesions induced by DON were mild villi atrophy and flattening and vacuolization of enterocytes. Overall, the jejunum lesional score in the DON group was 3-fold higher than the score in the control group (Fig 2B). Supplementation with *S. cerevisiae* boulardii reduced the effects of DON on the jejunum, and there was no statistically significant difference in the lesional score between this group and the score of the control group. Edema of the *lamina propria* and lymphatic dilatation remained the most frequent histological changes (Fig 2A). Still, administration of *S. cerevisiae* boulardii to piglets exposed to DON significantly reduced the severity of villi atrophy, and limited the flattening and vacuolisation of enterocytes. Also, there was no statistically significant difference in lesional scores between piglets only supplemented in yeast, and piglets exposed to DON and supplemented in yeast (Fig 2B).

In the liver, a 2.5-fold increase in the lesional score was observed in the DON-group compared to control (Fig 2C & 2D). The lesions mainly related to vacuolisation of the nucleus of hepatocytes, megalocytosis and apoptosis. The liver of the piglets supplemented with *S. cerevisiae* boulardii alone showed neither megalocytosis nor apoptosis (Fig 2C). However, this group presented a higher frequency of inflammatory infiltrate (57%) compared to other groups (control 30%, DON 40%). The inflammatory infiltrate consisted in lymphocytes. In addition, supplementation with *S. cerevisiae* boulardii of DON-exposed piglets reduced the occurrence of inflammatory infiltrate (12.5%), and overall, the lesional score in this group was similar to the score of the control group.

Histological examination of the kidney also revealed clear differences among the experimental groups (Fig 2E & 2F). The lesional scores of the piglet kidneys increased 3 fold upon exposure to DON, while the animals receiving *S. cerevisiae* boulardii alone did not differ from the control group. Conversely, the lesional scores of the piglets exposed to DON and supplemented with *S. cerevisiae* boulardii were similar to the control group. The most frequent lesions observed in piglets exposed to 3 mg/Kg DON for four weeks were kidney congestion (50%), and vacuolization of the cytoplasm (80%) and nucleus (40%) of tubular epithelial cells (Fig 2E). No vacuolisation of the nucleus was detected in piglets receiving *S. cerevisiae* boulardii, even when exposed to DON, and dramatic reduction in the frequency of cytoplasmic vacuolisation was confirmed in the piglets exposed to the mycotoxin and supplemented with *S. cerevisiae* boulardii (37.5%).
Taken together, these data demonstrate that supplementing the diet with *S. cerevisiae* boulardii was able restored the histological lesions induced by DON in the intestine, the liver and the kidney.

3.3. Supplementation with *S. cerevisiae* boulardii partially restores variation in the endogenous metabolites induced by DON

^{1}H NMR spectra of plasma analyses from a representative pig of each group (control, *S. cerevisiae* boulardii, DON, and DON+*S. cerevisiae* boulardii) are presented in Supplemental Figure 1. Thirty-four metabolites were identified in plasma NMR spectra, and 52 in aqueous liver extracts.
Figure 3 presents the score plots of the PLS-DA analysis for plasma and liver sample. The PLS-DA model performance parameters including several classification measures and permutation test results are provided in Supplemental Table 2. For plasma samples, a valid and robust PLS-DA model was adjusted (A=2 latent variables, R^2=62%, Q^2=0.56, misclassification rate was less than 25%, indicating a good classifier model, and the mean AUROC was 0.71, confirming the good classifier). The first dimension of the PLS-DA (vertical axis) clearly segregated the DON-treated group from the others, while the second dimension (horizontal axis) clearly discriminated the two groups supplemented with *S. cerevisiae* boulardii, and underlined the similarity between the profiles of the control and the DON+ *S. cerevisiae* boulardii groups (Fig 3A). A valid and robust model was also fitted for liver samples (A=2 latent variables, R^2=58.3%, Q^2=0.44). The score plot discriminated the control group from the other ones (dimension 1), but did not separate the DON and DON+ *S. cerevisiae* boulardii conditions (Fig 3B).
Table 3 summarises the metabolites identified in the NMR spectra which discriminated the four groups for the plasma or the liver sample analyses from the 4-groups PLS-DA model. Setting the VIP threshold at 0.8, 93 buckets corresponding to 22 metabolites discriminated the treatments in the plasma, and 41 buckets corresponding to seven metabolites discriminated the treatments in the liver. Comparison with published databases and two-dimensional (2D) ^{1}H-^{1}H COSY and 2D ^{1}H-^{13}C-HSQC NMR experiments unambiguously assigned 17 metabolites in plasma and six metabolites in liver samples, respectively.
In order to identify the metabolic pathways affected by exposure to 3 mg/kg DON or feed supplementation with *S. cerevisiae* boulardii at 4×10^{9} CFU/kg, the metabolites detected as discriminative between the DON and control conditions and the *S. cerevisiae* boulardii and control conditions in the plasma samples were implemented in the MetExplore metabolic network analysis tool. These metabolites were identified from a binary PLS-DA model followed by a univariate Mann-Whitney analysis (Supplemental figures S2 and S3, and Supplemental Table 3). The subnetwork of interest related to the DON-exposed piglets is displayed in Figure 4,

while Table 4 lists the significantly enriched metabolic pathways (right-tailed Fisher test with Bonferroni's multiple test correction) for DON condition and *S. cerevisiae* boulardii as well. As can be seen in the figure and table, exposure to DON primarily affected the biosynthesis of aminoacyl-tRNA and the metabolism of amino acids and 2-Oxocarboxylic acids. On the other hand, the yeast supplementation not only impacted the biosynthesis of aminoacyl-tRNA and the metabolism of amino acids, but it also affected the glycerophospholipid metabolism.

Figure 5 and figure 6 present, for the different conditions, the level of metabolites identified from binary PLS-DA followed by univariate Mann-Whitney analysis as being affected by DON exposure in plasma and liver tissue. In most cases, no significant difference was detected between the control and the DON+ *S. cerevisiae* boulardii conditions, thereby confirming that probiotic supplementation reversed the alteration of metabolite profile in plasma induced by exposure to DON.

Compared to the control, exposure to DON increased the glutamine concentration in liver and reduced the concentration of glucose, arginine, betaine and glycerophosphocholine (Table 3). The *S. cerevisiae* boulardii supplement fed to the piglets tended to normalise the level of glutamine in the liver, although it had no effect on the other metabolites (Fig 6).

4. Discussion

DON is one of the most notorious mycotoxins from the point of view of pig health. It presents the highest percentage of positive samples in the feed supply chain, and the highest ranges of contamination [32-34]. Although the strict enforcement of current regulations on maximum levels in feed and feedstuffs has solved the problem of the acute toxicity of DON and other mycotoxins, the residual subclinical doses can still have a significant impact on animal health and productivity, especially on young ones [35, 36].

In the experiment reported here, the dietary exposure of weanling piglets to 3 mg/kg DON for 28 days induced no significant clinical alteration nor significant change of plasmatic biochemical parameters. This outcome was expected since previous studies already showed that sub-chronic exposure up to 6 mg/kg DON did not modify the plasma biochemical profile [37-39]. By contrast, pig tissue histological alterations, especially at the intestinal level, have shown a higher sensitivity to mycotoxin exposure at doses considered realistic under enforcement of the current regulations pertaining to pig feed [40, 41]. Consequently, to analyse the effects of *S. cerevisiae* boulardii supplementation under realistic exposure to this feed contaminant, we investigated the histological lesions in the intestine, liver and kidney, as the pivotal organs in DON absorption, metabolism and excretion [1]. Additionally, a metabolomics study on plasma and liver sample was conducted to detect changes in metabolite levels that could be associated with non-physiological conditions, if any.

As expected, significant modifications appeared in the histology of the investigated organs in the DON group compared to the control group. The types and severity of liver and intestinal lesions reported in this study are consistent with previous observations on both organs, after a short-term exposure to DON at levels starting from 1.5 mg/kg, which confirms the higher sensitivity of the histological analysis for the sub-acute toxicity of DON and other mycotoxins [41, 42].

To gain insight into the effect of the modifications induced at the cellular level, metabolic ^{1}H-NMR fingerprints of the plasma and liver samples were generated and coupled with metabolic network modeling. Using only a ^{1}H-NMR platform is a limitation to cover all the metabolome. Nonetheless, significant evidences for metabolic profile disorder was detected in piglets exposed to 3 mg/Kg DON, in the absence of clinical signs and alteration of blood biochemistry

parameters. There is no universal technique covering the diverse range of analyte structures and polarities present in biological samples [43], and the development of tools for biochemical pathway analysis aims at building associations between the analytes retrieved from this spotty information and existing knowledge, in order to explain the link between changes in metabolite concentrations and the factor being studied [29, 43].

MetExplore network analysis of the ^{1}H-NMR fingerprints revealed that the biosynthesis of aminoacyl-tRNA and the metabolism of amino acids and 2-oxocarboxylic acids were the main metabolic pathways affected in piglets exposed to DON. Aminoacyl-tRNA synthesis is an ubiquitous cellular process that acts as an interface between nucleic acid and amino acid sequences, by providing the appropriate substrates for the ribosomal translation of messenger RNA during protein synthesis [44]. The impact on glucose and amino acids metabolism was previously identified in two studies on metabolomics profiling of urine, kidney and spleen of Kunming mice exposed to a subclinical dose of DON [45, 46]. The metabolism of 2-oxocarboxylic acids or alpha-keto acids highlighted by the metabolite network analysis also fit in the protein synthesis, as they are involved in transamination reactions which occurr during the biosynthesis and catabolism of several amino acids including valine, leucine and isoleucine [47]. Inhibition of protein synthesis is among the best characterised cellular effects of trichothecene mycotoxins on eukaryotic cells, and DON targets both the ribosomal and mitochondrial translation processes in particular [1, 48]. It is to the credit of the metabolomics tool used in this work that we were able to highlight in *in vivo* conditions the alteration to protein synthesis induced by the trichothecene mycotoxins which to date, has mainly been demonstrated in cell-scale experiments using *in vitro* assays [49, 50].

Several alterations were detected at the histological examination of the liver tissue of DON-exposed piglets, and an increase of a classical marker of hepatic dysfunction (AST) was observed, although the other transaminase ALT was not affected at the biochemistry analysis of plasma. On the other hand, metabolomics revealed a decrease of the level of betaine in the liver of the piglets exposed to DON, along with an increase of betaine metabolite sarcosine and methionine in their plasma. Betaine is a vital methylating agent that has been shown to protect the liver from a wide range of toxins such as chloroform, methotrexate, lipopolysaccharide, and carbon tetrachloride [51]. Betaine as a lipotrope, prevents the accumulation of fat in the liver by enhancing the methylation of phosphatidylethanolamine to form phosphatidylcholine [52]. As a result, administration of betaine reduces hepatic lipidosis with typical presence of multiple cytoplasmic vacuoles [51, 53]. Betaine also demonstrated inhibition of the pro-apoptotic mitochondrial pathway in the bile-induced apoptosis in hepatic tissue [54]. The apoptotic foci and vacuolization reported both in liver and kidney tissue of the DON-exposed piglets correlate the decrease of betaine level. Moreover, the elevated plasma concentration of the betaine metabolite sarcosine is consistent with the detected hepatic alterations since increase of betaine metabolites, especially sarcosine, is frequently seen in patients with chronic liver disease, and thought to result from the increased activity of the betaine homocysteine methyl transferase enzyme BHMT [55]. The hypothesis of an increased activity of BHMT and underlying hepatic alterations is finally supported by the higher levels of methionine evidenced in the plasma of DON-exposed piglets. Indeed, a metabolic trans-methylation pathway closely interconnects betaine and methionine which intersect at the BHMT-catalysed formation of methionine from homocysteine [56].

Providing a supplement of *S. cerevisiae* boulardii to piglets exposed to DON considerably reduced the severity of the histological lesions in the intestine, liver and kidney. This is not the first report of beneficial properties of a probiotic strain on mycotoxin-induced tissue alterations.

A *Lactobacillus plantarum* strain, with no biotransformation ability toward mycotoxins, was shown to protect chickens against the subclinical toxicity of DON [10, 57]. *L. rhamnosus* was also shown to limit intestinal tissue alteration induced by DON in a pig jejunal explant model [8]. *S. cerevisiae* boulardii has demonstrated some potential in the treatment or prevention of ulcers induced by non-steroidal anti-inflammatory drugs in a rat model of ibuprofen-induced gastric ulcer [58]. Using the previously reported pig jejunal ex-vivo model, we also observed that *S. cerevisiae* boulardii significantly reduced the overall impact of DON on the intestinal transcriptome, reversing some prototypical signaling pathways linked to inflammation and immunity, and reducing the burden of the global DON-induced oxidative stress [17]. These results correlated previous findings indicating that *S. cerevisiae* boulardii produces a low molecular weight water-soluble and heat stable factor termed SAIF, which has an anti-inflammatory effect [16, 59]. Based on reports indicating that *S. cerevisiae* boulardii, otherwise well-characterised as a gastro-intestinal condition enhancer, can also attenuate the D-galactosamine-induced liver injury and ameliorate the carbon tetrachloride-induced liver fibrosis in rodents [19, 20], we hypothesised that the protective effects of the yeast could extend to DON systemic toxicity.

Beside glycerophospholipid metabolism, we reported that *S. cerevisiae* boulardii supplementation to the piglets impacted two other metabolic pathways that were also targeted by DON, the biosynthesis of aminoacyl-tRNA and the metabolism of amino acids. Interestingly, phenylalanine, tyrosine and tryptophan biosynthesis, and glycerophospholipid metabolism were the significantly enriched KEGG pathways identified from the transcriptomic analysis of the intestinal effects of another probiotic strain that demonstrated efficacy against DON-induced tissue alteration [57]. Aside from the already discussed metabolism of amino acids, the seemingly pivotal role of glycerophospholipid metabolism in the anti-DON activity of these probiotic supplementations might be connected with the antioxidant and anti-inflammatory properties that justify the dietary supplementation of glycerophospholipids as a potential strategy to counteract aging of cerebral structures [60]. At least two potential pathways through which supplementation with glycerophospholipids may modulate the activity or concentrations of ROS and endogenous antioxidants have been identified. The first pathway involves the upregulation of transcription factors associated with the synthesis of endogenous antioxidants especially by ω3-PUFA-containing glycerophospholipids [61, 62]. The second pathway is by downregulating the activity of select enzymes that produce ROS, especially the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [63]. Likewise, glycerophospholipids, as significant sources of choline, can stimulate the cholinergic anti-inflammatory pathway, as well as they can influence inflammation through modifying the fatty acid composition of cell membranes, including inflammatory cells [64-66].

Feed supplementation with *S. cerevisiae* boulardii led to the almost total restoration of the plasma metabolic profile. By contrast, the effect of the probiotic supplementation on the liver metabolome was marginal, indicating that the toxicity of the mycotoxin was mitigated, but not abolished. Feeding *S. cerevisiae* boulardii as a supplement to rats exposed to carbon tetrachloride resulted in significant changes in intestinal permeability, and the protective effect of the yeast was partially attributed to the improvement of the toxic-induced alteration of the barrier function [19]. Both paracellular and transcellular paths have been reported for the intestinal transfer of DON to the systemic compartment, though DON transiting via transcellular pathmay be substrate to efflux systems involving the P-glycoprotein, and the multi-drug resistance-associated protein 2 [1, 67, 68]. Moreover, DON transport from the apical to the basolateral side of Caco-2 cells and, inversely, from the basolateral to the apical side have been

shown to be directly proportional to its initial concentration as well as to the duration of the incubation, suggesting that, in contrast to the paracellular flux, the overall contribution of the transcellular passage, may be limited [69]. At the cellular level, disintegration of the tight junction protein network formed by claudins and their binding partner ZO-1 resulting in a loss of barrier function has been shown to be one of the main *in vivo* effects of DON on the intestine [70, 71]. Hence, one consequence of the DON-induced alteration of the tight junction protein integrity could be increased systemic exposure to the mycotoxin and the subsequent detrimental effects on internal organs.

The administration of the yeast to broilers improved the barrier function via up-regulation of the tight junction proteins occludin, claudin 2 and claudin 3 gene expression [72]. The tight junction protein complex is attached to a perijunctional actomyosin ring whose contraction regulates the tight junction barrier function [73]. *S. cerevisiae* boulardii has been shown to inhibit the phosphorylation of the myosin II regulatory light chain (MLC) induced by enterohemorrhagic *Escherichia coli* infections [74]. Phosphorylation of MLC is the biochemical marker for actomyosin contraction.

Major intestinal inflammatory diseases such as Crohn's disease, ulcerative colitis, celiac disease, and irritable bowel syndrome are associated with the decreased expression of sealing claudins, and the production of different cytokines is thought to be responsible for the downregulation of the claudins in inflamed intestinal mucosa [75]. Ultimately, the possibility cannot be excluded that the DON-induced breakdown of the tight junction integrity is a consequence of its intestinal pro-inflammatory effect [76, 77]. One hypothesis that deserves investigation is that, by reversing the DON-induced intestinal inflammation, supplementing the diet of piglets exposed to DON with *S. cerevisiae* boulardii restores tight junction integrity, thereby limiting the exposure of the internal organs to the mycotoxin and subsequent toxicity.

This study did not analyze the effect of DON and *S. cerevisiae* boulardii on the microbiota of the exposed piglets. A previous work indicated that a probiotic *Lactobacillus plantarum* strain conteracts the DON-induced effects in broilers by reducing the damages observed in intestinal morphology and intestinal barriers, along with an increase of the abundance of beneficial bacterium and the regulation of the balance of gut microbiota [56]. However, whether an interaction exists between those components of the reported anti-DON effect is still unclear. Especially regarding the administration of *S. cerevisiae* boulardii, the fact that at least two previous studies pinpointed the microbiome effects of the yeast administration in prevention of the toxicity of xenobiotics [19, 20] advocates for a thorough investigation of the possible interplay between host tissue healing and probiotic-induced microbiota modification. This perspective is currently under investigation in our lab.

To conclude, the aim of the present study was to investigate the effects of supplementation of a S. *cerevisiae* boulardii strain in feed as a strategy to alleviate the subclinical effects of exposure to DON in piglets. Although neither clinical signs nor significant modifications of the classical blood biochemistry were detected, exposure of piglets to DON at 3 mg/kg of feed for 28 days was seen to induce histological alterations in organs which play a key role in the mycotoxin metabolism. Indications of alteration in protein metabolism were also provided by ^{1}H-NMR analysis of two different biological matrices. Supplementation with the yeast strain clearly prevented tissue lesions in piglets exposed to DON. Moreover, no significant metabolic modification was found in the plasma, and persisting alteration of the liver metabolic profile was limited. Taken together, these observations outline that S. *cerevisiae* boulardii supplementation can increase the piglet resilience to a subclinical DON challenge.

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Table 1: Basal diet formulation and nutrient contents

Ingredient	*g/kg*
Wheat	475
Soybean meal	243
Barley	229
Calcium phosphate	11.2
Calcium carbonate	10
Vitamin and mineral premix[1]	5
Vegetable oil	14
Sodium chloride	4
Phytase	0.1
Lysine	4.65
Methionine	1.65
Threonine	1.95
Tryptophan	0.45
Proximate analysis	*g/kg*
Starch	4768
Crude protein	2183
Crude fiber	375
Calcium	105
Phosphorous	65
Potassium	87
	MJ/kg
Net energy	156

[1] Vitamin A. 2 000 000 IU/kg; vitamin D_3. 400 000 IU/kg; vitamin E. 4000 mg/kg; vitamin C. 8000 mg/kg; vitamin B_1. 400 mg/kg; vitamin K_3. 400 mg/kg; iron. 20 000 mg/kg; copper. 4000 mg/kg; zinc. 20 000 mg/kg; manganese. 8000 mg/kg.

Table 2: Effects of DON-contaminated diets and feed supplementation with S. cerevisiae boulardii on plasma biochemistry parameters

Biochemical parameters	Animal diet				*P*- value		
	Control	*S. cerevisiae* boulardii	DON	DON + *S. cerevisiae* boulardii	Diet	Day	Diet x Day
Albumin (µmol/L)	472	449	449	465	0.141	0.000	0.901
ALP (U/L)	241[a]	198[b]	245[a]	237[a]	0.001	0.060	0.254
ALT (U/L)	48.2[a]	47.9[a]	53.9[b]	51.3[ab]	0.031	0.031	0.626
AST (U/L)	71.2	92.5	88.2	103	0.403	0.817	0.687
Calcium (mmol/L)	2.76	2.68	2.67	2.72	0.054	0.061	0.706
Cholesterol (mmol/L)	2.09	2.08	2.02	2.09	0.626	0.000	0.592
Creatinin (µmol/L)	72.8	70	68.2	73	0.622	0.000	0.344
Glucose (mmol/L)	5.93	5.91	6.15	5.39	0.793	0.000	0.104
Lactate (mmol/L)	11	9.52	9.08	9.54	0.408	0.104	0.378
LDH (U/L)	1204	1271	1243	1294	0.344	0.366	0.657
Magnesium (mmol/L)	0.92	0.90	0.87	0.94	0.706	0.000	0.125
Phosphorous (mmol/L)	2.84	2.77	2.74	2.92	0.315	0.000	0.514
Total proteins (g/L)	54.5	55. 7	53	54.2	0.810	0.000	0.937
Triglycerides (mmol/L)	0.63	0.65	0.61	0.65	0.291	0.018	0.854
Urea (mmol/L)	2.37[a]	2.53[a]	1.82[b]	2.28[a]	0.009	0.021	0.099

Data are mean ± SD for 8 to 10 animals. Means in a row with different letter differ ($P < 0.05$).

Table 3: Endogenous metabolite variations observed in the DON, S.c. boulardii and DON+S.c. boulardii groups compared to the control group and in the DON group compared to DON+S.c. boulardii group in aqueous liver extracts and plasma samples

Metabolites	**^{1}H NMR chemical shifts (ppm)**	***P*-value FDR**	**CTRL → DON**	**CTRL → S.c. boulardii**	**CTRL→DON + S.c.boulardii**	**DON →DON + S.c. boulardii**
Liver						
Betaine	3.27(s) ; 3.90(s)	0.0008	↘		↘	
Glucose	3.25(dd,7.3 and 7.9); 3.42(m); 3.47(m); 3.51(m); 3.54(m); 3.72(m); 3.73(m); 3.77(m); 3.84(m); 3.90(m); 4.65(d,8); 5.24(d,3.8)	0.0013	↘	↘	↘	
Glutamine	2.14(m); 2.46(m); 3.78(t,6.2)	0.0319	↗			
Glycerophosphocholine	3.23(s); 3.62(m); 3.68(m);3.89(m); 3.94(m); 4.33(m)	0.0009		↗	↗	
Unknown	2.76 (s)	0.0371	↗			
Plasma						
Creatine	3.04(s); 3.93(s)	0.047		↗		
Glucose	3.25(dd,7.3 and 7.9); 3.42(m); 3.47(m); 3.51(m); 3.54(m); 3.72(m); 3.73(m); 3.77(m); 3.84(m); 3.90(m); 4.65(d,8); 5.24(d,3.8)	0.0006	↗	↗		↘
Glutamine	2.14(m); 2.46(m); 3.78(t,6.2)	0.005	↗			
Glycerophosphocholine	3.23(s); 3.62(m); 3.68(m);3.89(m); 3.94(m); 4.33(m)	0.0098		↘		
Lactate	1.33(d,6.9); 4.12(q,6.9)	0.0001	↘			↗
Lipids	0.88(m) ; 1.28(m) ; 5.31(m)	0.034		↘		
Lysine	1.45(m); 1.52(m); 1.73(m); 1.91(m); 3.02(t, 7.5)	0.022		↗		
Phenylalanine	3.14(dd,14.5 and 7.8) ; 3.29(dd,5.2 and 14.5) ; 4.00(dd,5.2 and 7.8) ; 7.33(d,7.3); 7.38(t,7.3); 7.43(t,7.3)	0.007		↗		
Unknown	2.04	0.038		↗		
Valine	0.99(d,6.9); 1.05(d,6.9); 2.28(m); 3.62(d,4.3)	0.003		↗		

Chemical shifts (ppm) are relative to TSP (^{1}H, δ, 0 ppm). Multiplicity of signals is indicated in brackets: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quadruplet and m, multiplet. Values into brackets are ^{1}H-^{1}H splits (Hz) in the case where these are clearly resolved. "↗" and "↘" indicate that the relative abundance increases and decreases respectively, in the treated group compared to control group, and in the DON S.c. boulardii group compared to the DON group.

Table 4: Metabolic pathways significantly modulated in the plasma of DON-exposed or yeast-supplemented piglets

Pathway name	Number of mapped metabolites	Coverage (%)	Adjusted *P*-value
Deoxynivalenol			
Aminoacyl-tRNA biosynthesis	6	12.77	1.47×10^{-5}
Biosynthesis of amino acids	6	9.52	4.41×10^{-5}
Alanine, aspartate and glutamate metabolism	4	12.12	7.81×10^{-4}
Glycine, serine and threonine metabolism	3	9.38	9.52×10^{-3}
Valine, leucine and isoleucine biosynthesis	2	25	0.011
2-Oxocarboxylic acid metabolism	2	9.52	0.046
***Saccharomyces cerevisiae* boulardii**			
Glycerophospholipid metabolism	3	7.89	0.029
Glycine, serine and threonine metabolism	3	9.38	0.035
Aminoacyl-tRNA biosynthesis	3	6.38	0.036

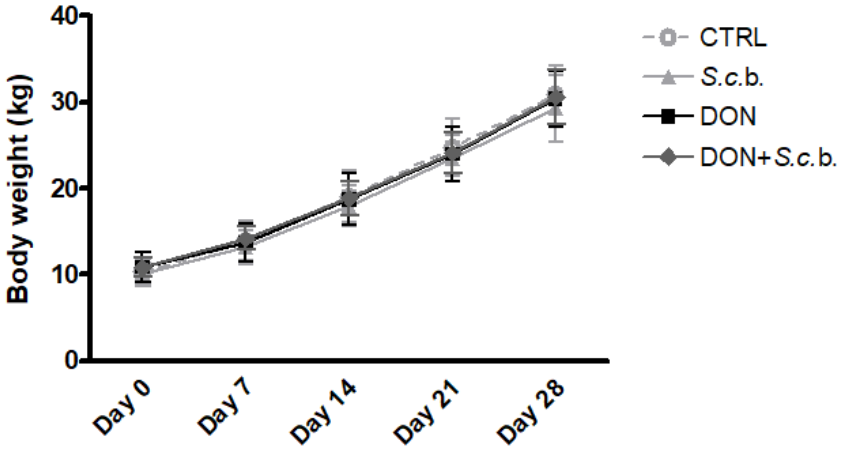


Figure 1: Effects of DON and *S. cerevisiae* boulardii supplementation on piglets' growth
Weight gain was recorded weekly on piglets fed *ad libitum* four different diets for 28 days: basal diet (CTRL); basal diet supplemented with *S. cerevisiae* boulardii CNCM I-1079 (4x109 CFU/kg feed), DON-contaminated basal diet (2.82 mg DON/kg feed,) supplemented or not with *S. cerevisiae* boulardii CNCM I-1079. Data are expressed as mean weight ± SD for 8 to 10 animals.

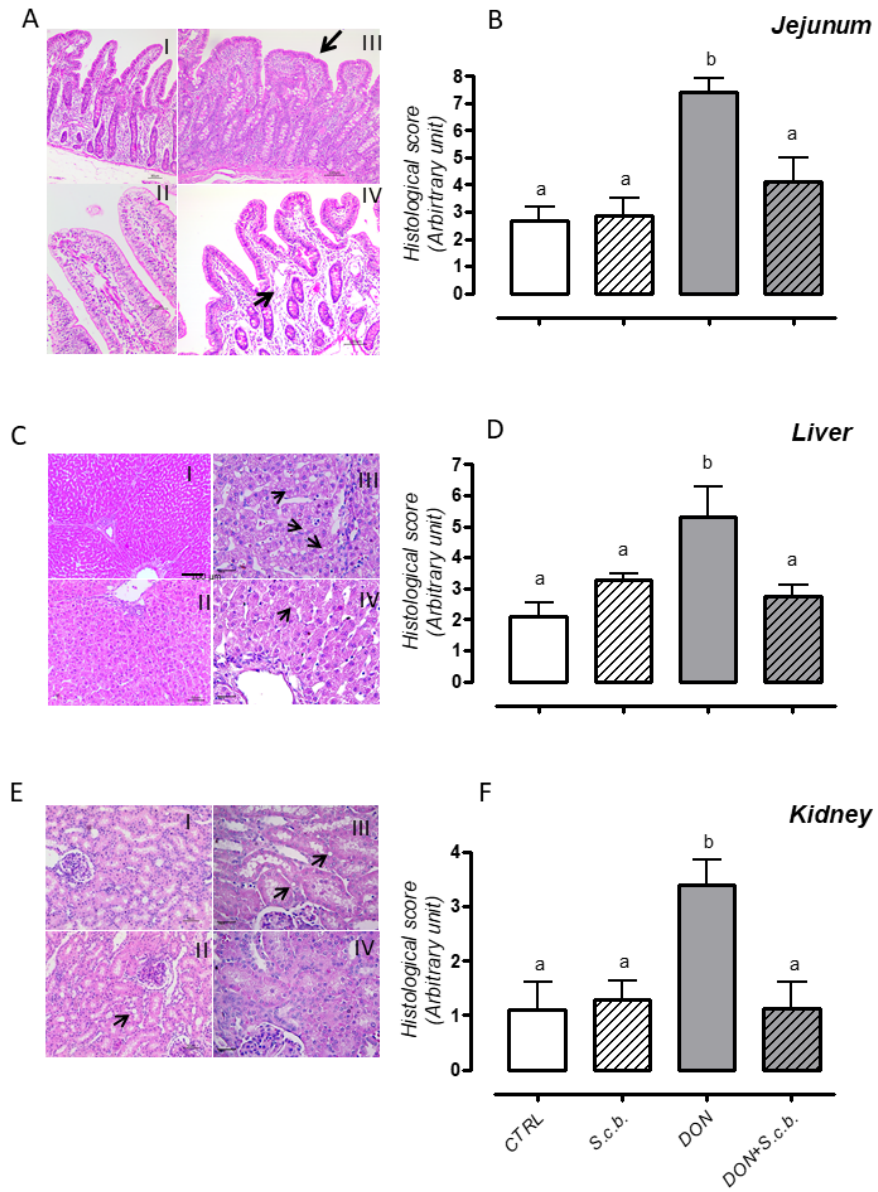


Figure 2: Effect of a 28-day exposure to DON-contaminated diets with and without *S. cerevisiae* boulardii on the jejunum, liver and kidney histology.

The piglets received one of four dietary treatments: basal diet (CTRL); basal diet supplemented with *S. cerevisiae* boulardii (S.c.b), DON-contaminated basal diet (DON) supplemented or not with *S. cerevisiae* boulardii (DON+S.c.b).

Jejunum, pig. A. I- Control. Normal jejunum. HE. Bar 80 µm. II- *S. cerevisiae* boulardii. Mild enterocyte cytoplasmic vacuolation. HE. Bar 50 µm. III- DON. Villi atrophy and fusion (arrow). HE. Bar 100 µm. IV- DON+ *S. cerevisiae* boulardii. Oedema of the *lamina propria* (arrow). HE. Bar 100 µm. B. Jejunum histological score of piglets under the different treatments.

Liver, pig. C. I- Control. Normal liver. II- *S. cerevisiae* boulardii. Mild trabecular hepatocyte disorganization. HE. Bar 50 µm. III- DON. Moderate hepatocyte cytoplasmic vacuolation (arrows). HE. Bar 30 µm. IV- DON+ *S. cerevisiae* boulardii. Mild hepatocyte cytoplasmic vacuolation (arrow). HE. Bar 30 µm. D. Liver histological score of pigs under the different treatments.

Kidney, pig. E. I- Control. Normal kidney. HE. Bar 50 µm. II- *S. cerevisiae* boulardii. Mild epithelial tubular vacuolation (arrow). HE. Bar 50 µm. III- DON. Moderate tubular epithelial cell nuclear vacuolation (arrows). HE. Bar 30 µm. IV- DON+ *S. cerevisiae* boulardii. Normal appearance of epithelial tubular cells. HE. Bar 30 µm. F. Kidney histological score of pigs under the different treatments.

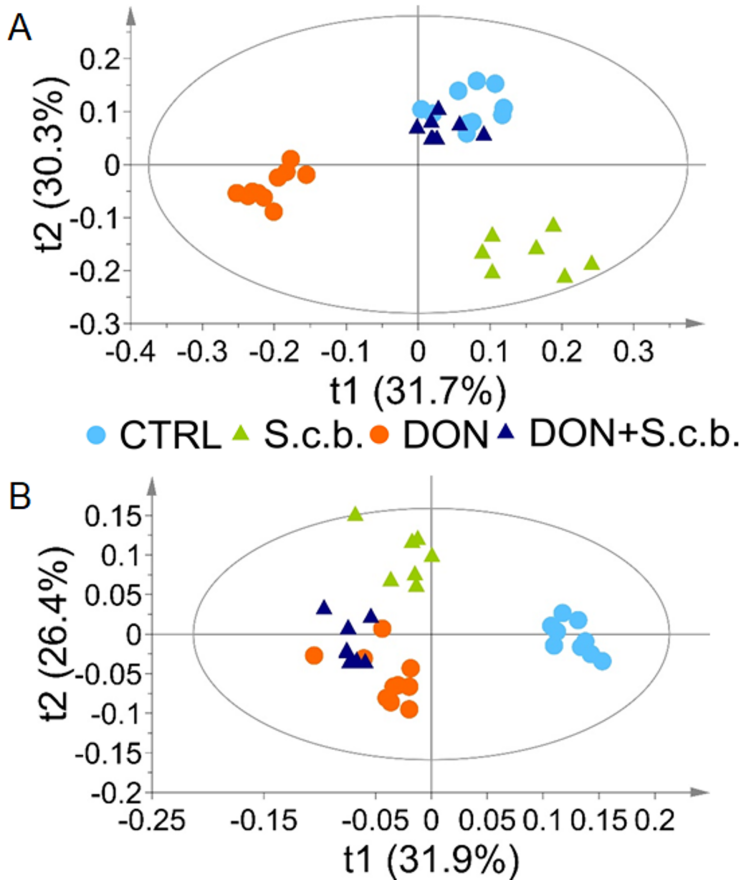


Figure 3: Two-dimensional partial least square discriminant analysis (PLS-DA) scores plot of integrated ^{1}H-NMR spectra.

Each symbol represents an observation (animal), projected onto first (horizontal axis) and second (vertical axis) PLS-DA latent variables. Treatment groups are distinguished by different colours and symbols: White dot: Controls, white triangle: *S. cerevisiae* boulardii, dark grey dot: DON, dark grey triangle: DON+ *S. cerevisiae* boulardii. The black ellipse represents the 95% confidence interval, which is drawn using Hotelling's T2 statistic. A. Plasma samples (A = 2, R^2 = 62.0%, Q^2 = 0.576). B. Liver samples (A = 2, R^2 = 58.3%, Q^2 = 0.441).

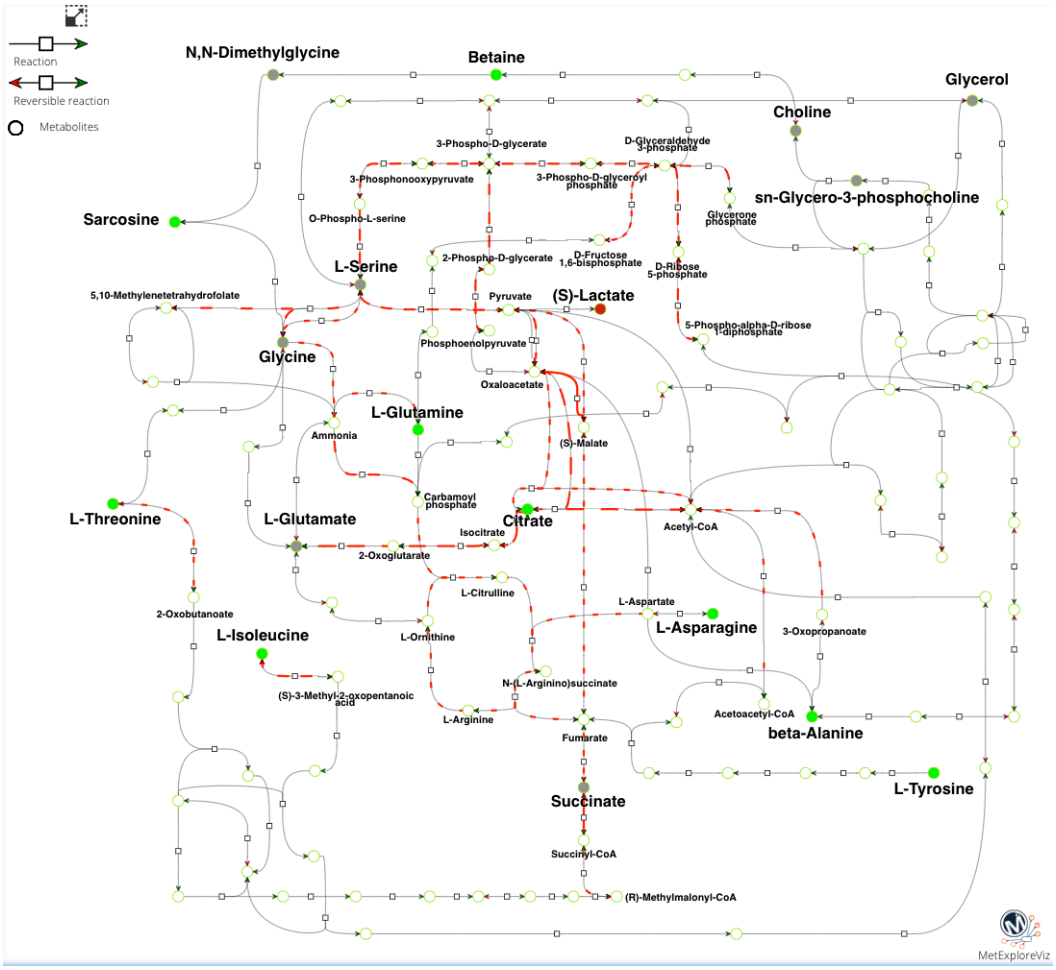


Figure 4: Metabolic pathways modulated by DON.

Genome scale metabolic network analysis was performed from plasma NMR fingerprinting. Circles represent metabolites and rectangles represent reactions. Metabolites identified as discriminant in the DON group compared to the control group according to the PLS-DA model are in green (increased concentration) or red (decreased concentration). Non-discriminant metabolites identified by NMR analysis are in grey. Dashed lines correspond to reactions belonging to either biosynthesis of amino acids, carbon metabolism and 2-oxocarboxylic acid metabolism. Width corresponds to the number of pathways to which each reaction belongs (the solid line means they belong to these three pathways). The label of a metabolite is only given if it belongs to at least one of the three pathways or is a metabolite identified by NMR. To obtain all the labels, readers are invited to load the Json file provided in supplementary material.

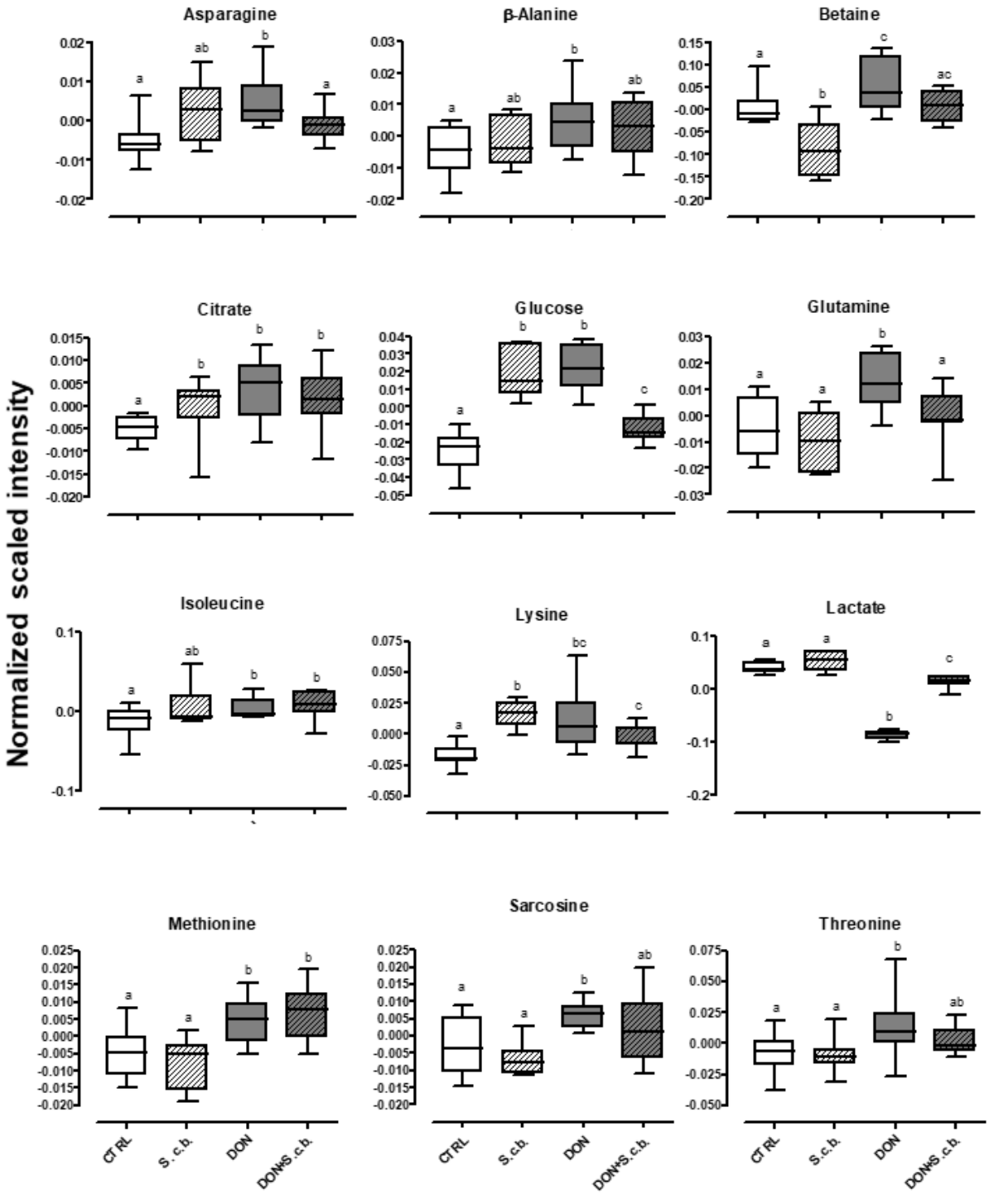


Figure 5: Effects of dietary exposure to DON and *S. cerevisiae* boulardii supplementation on the plasma level of selected metabolites

Plasma samples were collected in piglets fed four different diets for 28 days: (A) control, basal diet; (B) *S. cerevisiae* boulardii, basal diet supplemented with *S. cerevisiae* boulardii CNCM I-1079 ($4x10^9$ CFU/kg feed); (C) DON, DON-contaminated basal diet (2.82 mg DON/kg feed,); and (D) DON+*S. cerevisiae* boulardii, DON-contaminated basal diet supplemented with *S. cerevisiae* boulardii. Data are from 8 to 10 animals.

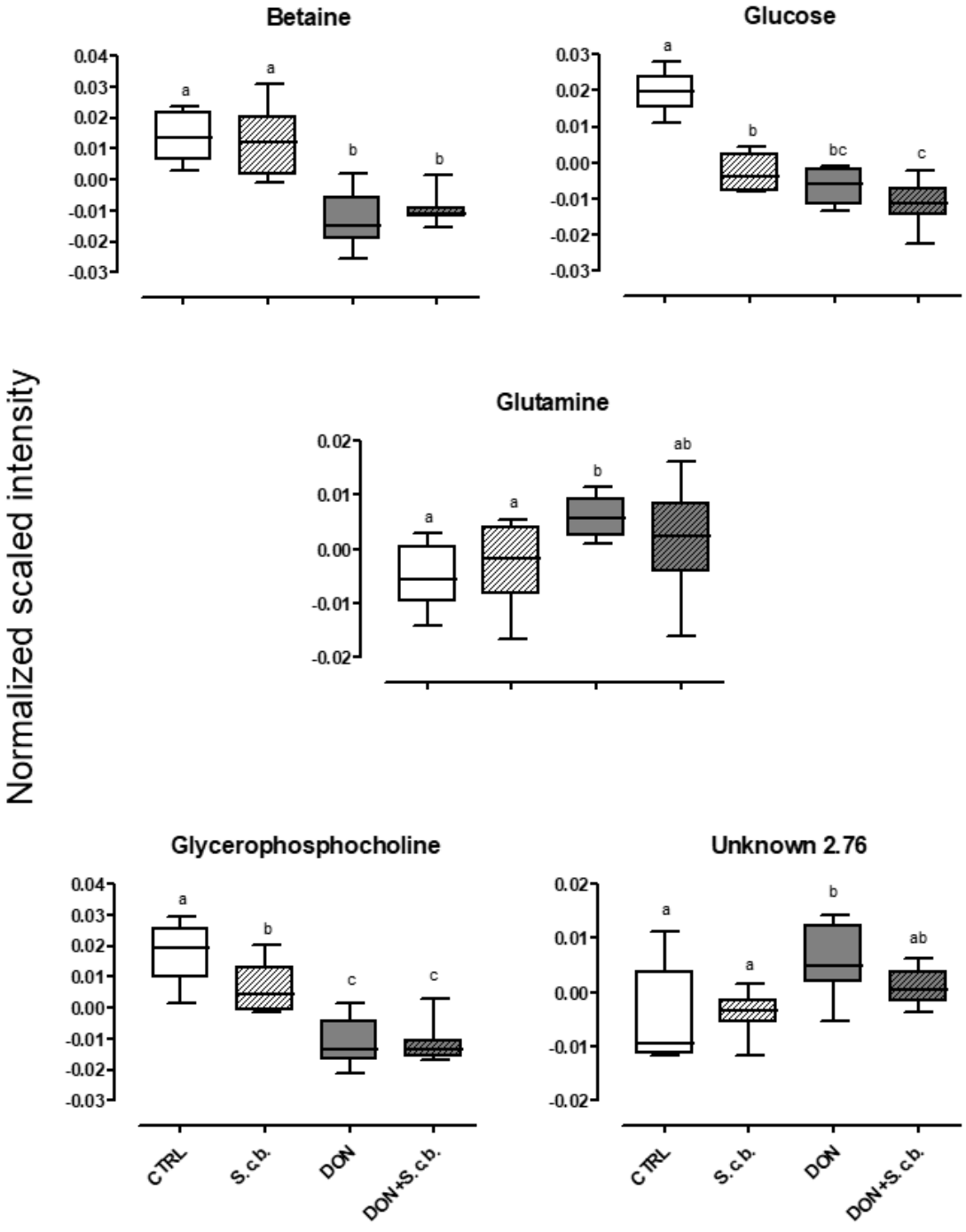
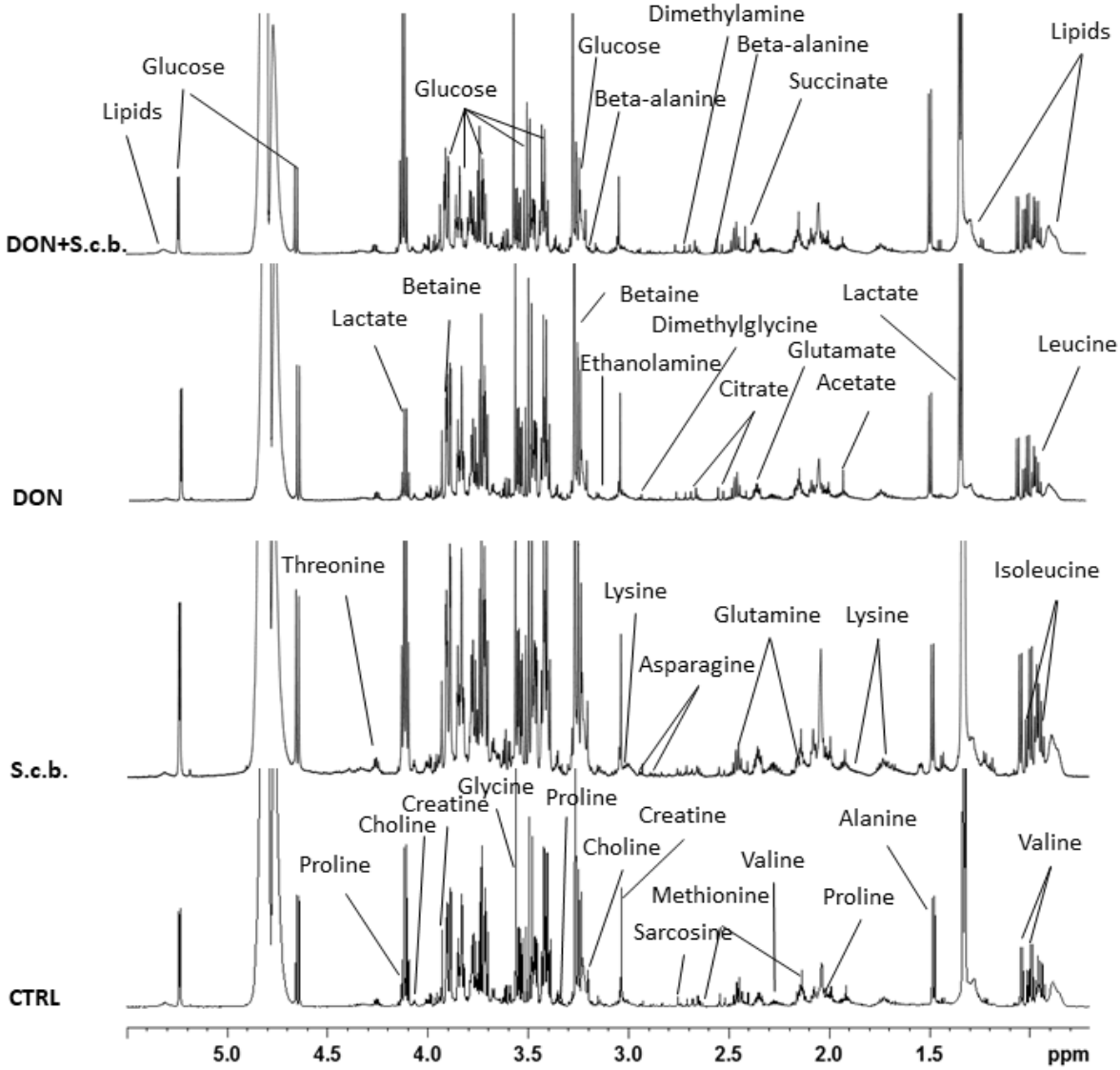
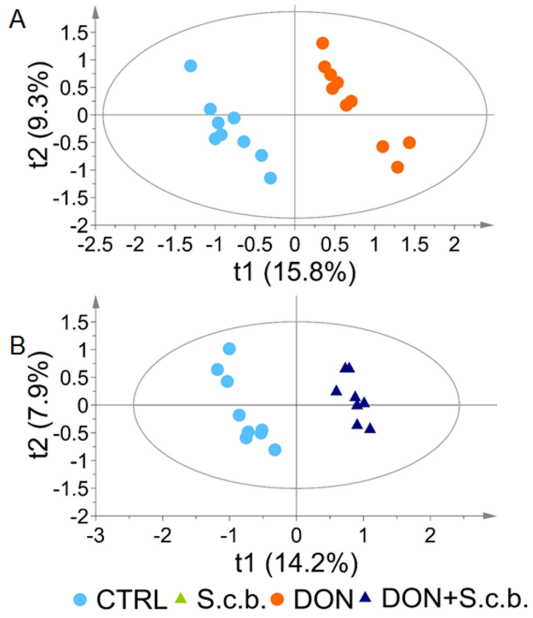


Figure 6: Effects of dietary exposure to DON and *S. cerevisiae* boulardii supplementation on the level of selected metabolites in the hepatic tissue.
Liver samples were collected in piglets fed four different diets for 28 days: (A) control, basal diet; (B) *S. cerevisiae* boulardii, basal diet supplemented with *S. cerevisiae* boulardii CNCM I-1079 ($4x10^9$ CFU/kg feed); (C) DON, DON-contaminated basal diet (2.82 mg DON/kg feed,); and (D) DON+*S. cerevisiae* boulardii, DON-contaminated basal diet supplemented with *S. cerevisiae* boulardii. Data are from 8 to 10 animals.

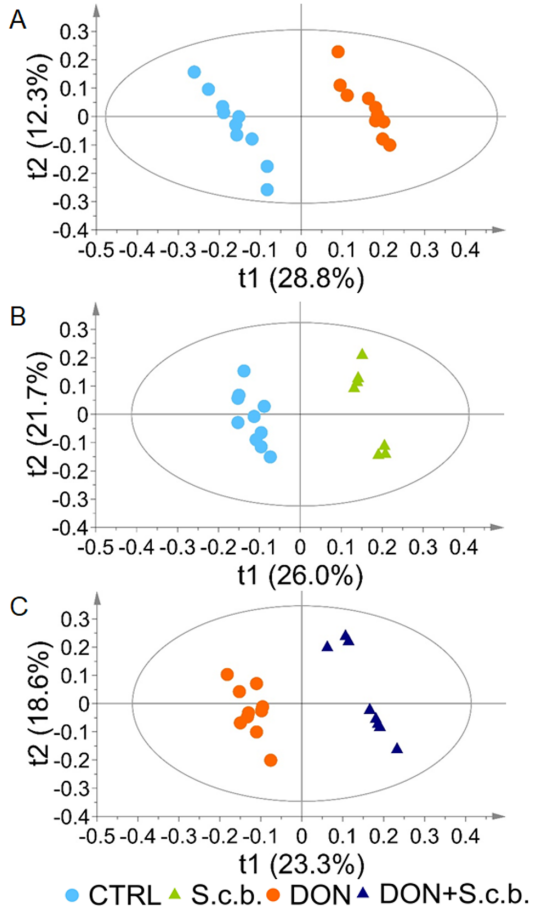


Supplemental Figure 1: Representative 600 MHz 1H CPMG NMR spectra (δ5.5-0.7 ppm) of piglet plasma samples.

Plasma samples were collected in piglets fed four different diets for 28 days: (A) control, basal diet; (B) *S. cerevisiae* boulardii, basal diet supplemented with *S. cerevisiae* boulardii CNCM I-1079 ($4x10^9$ CFU/kg feed); (C) DON, DON-contaminated basal diet (2.82 mg DON/kg feed); and (D) DON+*S. cerevisiae* boulardii, DON-contaminated basal diet supplemented with *S. cerevisiae* boulardii.



Supplemental Figure 2



Supplemental Figure 3