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RESEARCH PAPER

Protective role of cells and spores of *Shouchella clausii* SF174 against fructose-induced gut dysfunctions in small and large intestine

Anella Saggese[a,#], Valentina Barrella[a,b,#], Angela Di Porzio[a], Antonio Dario Troise[c], Andrea Scaloni[c], Luisa Cigliano[a,e], Giovanni Scala[a], Loredana Baccigalupi[b,d,e], Susanna Iossa[a,b,e], Ezio Ricca[a,e,*], Arianna Mazzoli[a]

[a] *Department of Biology, University of Naples Federico II, Naples, Italy*
[b] *NBFC, National Biodiversity Future Center, Palermo, Italy*
[c] *National Research Council, Proteomics, Metabolomics & Mass Spectrometry Laboratory, Institute for the Animal Production System in the Mediterranean Environment, Portici Naples, Italy*
[d] *Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy*
[e] *Task Force on Microbiome Studies, University of Naples Federico II, Napoli, Italy*

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Abstract

The oral administration of probiotics is nowadays recognized as a strategy to treat or prevent the consequences of unhealthy dietary habits. Here we analyze and compare the effects of the oral administration of vegetative cells or spores of *Shouchella clausii* SF174 in counteracting gut dysfunctions induced by 6 weeks of high fructose intake in a rat model. Gut microbiota composition, tight junction proteins, markers of inflammation and redox homeostasis were evaluated in ileum and colon in rats fed fructose rich diet and supplemented with cells or spores of *Shouchella clausii* SF174. Our results show that both spores and cells of SF174 were effective in preventing the fructose-induced metabolic damage to the gut, namely establishment of "leaky gut", inflammation and oxidative damage, thus preserving gut function. Our results also suggest that vegetative cells and germination-derived cells metabolize part of the ingested fructose at the ileum level.

Keywords: Fructose; Gut dysfunction; Probiotics; Spores; Cells; *Shouchella clausii*; Inflammation; Oxidative stress.

1. Introduction

Nowadays it is widely recognized that a correct diet is essential to ensure the maintenance of a health status. According to the World Health Organization [1], sugars should represent less than 10% of the total energy intake and be less than 5% to gain additional health benefits. Despite such clear indications, people in the Western world have exponentially increased their sugar consumption [2], mostly because of food- and drink-added sweeteners, present in over 70% of processed foods and accounting for approximately one-sixth of the total energy intake [3]. The major component of such added sweeteners is fructose [4] a common monosaccharide abundantly found in fruit. In the body, the excess of fructose causes oxidative stress [5], alters the intestinal environment by acting on the composition of the gut microbiota and on gut barrier permeability, inducing endotoxemia and systemic inflammation [6], thus leading to the development of nonalcoholic fatty liver disease, cardiometabolic syndrome [7], hypertension and diabetes [8], all symptoms of the so-called metabolic syndrome.

The gut microbiota plays a central role in the intestinal well-being, and this opens to a series of possible microbiota-directed intervention strategies, including the oral administration of prebiotics and/or probiotics, to reduce intestinal inflammation and damages [9].

The oral use of probiotics is generally well accepted because of the capacity of some bacteria to reduce inflammation markers and protect the integrity of the gut barrier [10]. Several species of the *Bifidobacterium, Lactobacillus, Bacillus* and *Saccharomyces* genera have long been used as commercial probiotics [11,12]. In this context, the use of *Bacillus*-based probiotics is peculiar, since all these commercial products do not contain live cells but metabolically quiescent (endo)spores [12]. Ingested spores safely transit the gastric barrier and reach the intestine, where some of them germi-

* Corresponding author at: Edificio 7, Via Cintia - I-80126 Napoli, Italy.
E-mail address: ezio.ricca@unina.it (E. Ricca).
# These authors contributed equally to the work.

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nate in the small intestine originating vegetative cells, able to grow and to sporulate again in the terminal part of the gastro-intestinal tract [13,14,15]. Therefore, upon spore ingestion both spores and germination-derived cells are present in the intestine and both cell forms interact with intestinal and immune cells. *Bacillus* cells are known to contribute to the normal development of the gut immune system [16], produce cytoprotective molecules [17] and protect the host from enteropathogens [18]. On the other end, spores are sensed by the murine immune system [19] and directly contact human epithelial cells inducing the nuclear translocation of the transcriptional factor Nrf-2 that, in turn activates stress-response genes [20]. In addition, it has been shown that spores modulate the microbial composition of the gut, favouring the presence of beneficial bacteria such as *Akkermansia muciniphila, Faecalibacterium prausnitzii* and various species of the *Bacteroidetes* genus [21,22]. Several *Bacillus* species are commonly used as probiotics. These include *B. clausii*, a species recently renamed *Shouchella clausii* [23] and herein referred to as *S. clausii*. This organism has a long history as a commercial probiotic [18], it has been shown to survive to simulated gastrointestinal conditions [15], inhibit enteropathogen and viral infections [24,25], enhance gut barrier functions and reduce inflammation, thus contributing to gut homeostasis [26]. Some *S. clausii* strains have also been tested in clinical studies as extensively reviewed by Lopetuso et al. [27] and Ghelardi et al. [18]. In the light of the above considerations, we analyzed and compared the effects of the oral administration of vegetative cells or spores of SF174, a *Bacillus* strain belonging to the *S. clausii* species in counteracting the fructose-induced alterations in the gut of young rats. *S. clausii* SF174, isolated from an ileal biopsy of a healthy human volunteer [28], was shown to survive gastric conditions in the spore form [28] and to have probiotic potentials [29].

2. Materials and methods

2.1. Bacterial strain and spore preparation and purification

S. clausii SF174 cells were grown aerobically for 16 h at 37°C in LB medium or in a modified S7 minimal medium: 50 mM morpholine-propanesulfonic acid (MOPS) (adjusted to pH 7.0 with KOH), 10 mM $(NH_4)_2SO_4$, 5 mM potassium phosphate (pH 7.0), 2 mM $MgCl_2$, 0.9 mM $CaCl_2$, 50 μM $MnCl_2$, 5 μM $FeCl_3$, 10 μM $ZnCl_2$, 2 μM thiamine hydrochloride, casamino acids 0.02%, LB 0.1%. The culture was centrifuged (5000xg for 10 min at room temperature) and lyophilized. Cells of SF174 were induced to sporulate at 37°C in Difco Sporulation Medium (DSM) [30] with vigorous shaking, for 30 hours. Spores were harvested by centrifugation (10 min; 10000xg), washed three times with distilled water and purified as described before [31,32]. Cleaned spores were stored at -20°C in water. Cells and spores count was determined by serial dilution and plate counting.

2.2. Animals and treatments

All animal experiments were authorized by Italian Health Ministry (137/2022-PR) and approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples "Federico II". The procedures used in this work observe the animal ethics principles and regulations of the Italian Health Ministry. The authors ensured that all steps were taken to minimize the pain and suffering of the animals.

Male Wistar rats (Charles River, Calco, Lecco, Italy) aged 30 days were caged in a temperature-controlled room (23 ± 1°C) with a 12 h light/dark cycle (06:30–18:30 h). Rats were divided into four groups of 8 rats each. The first group was fed a control diet (Co), while the other three groups were fed a fructose diet alone (F) or in combination with the daily administration of 0.5 mL of a 10% sucrose solution containing 5×10^9 colony forming units (CFU) of *Shouchella clausii* cells (c174) or 5×10^9 CFU of spores (sp174) (Supplementary Fig. S1). Co and F rats received the same amount of sucrose solution without probiotics. Sucrose solution with or without probiotics was presented by an operator every day at the same hour through a needless syringe and voluntarily consumed by rats. The amount of sucrose administered daily is negligible in terms of amount and energy content and was given to all animal groups. In fact, the daily amount of sucrose used for probiotic administration corresponds to 50 mg and 0.84 kJ. This amount represents 0.25% of the daily energy intake of rats and 0.42% of the daily intake of carbohydrates.

The diet treatment and probiotic administration were carried out for 6 weeks. The composition of the two diets is shown in Supplementary Table S1. Body weight and food intake were monitored daily, and no changes were evident between the four groups of rats during and at the end of the 6 weeks of treatment (Supplementary Fig. S2).

At the end of the experimental period, the rats were anesthetized with sodium pentothal (40 mg kg^{-1} intraperitoneal, i.p.) and euthanized by decapitation. Proximal ileum and colon were cleaned of their contents with a sterile 1% phosphate-buffered saline (PBS) solution. Proximal ileum and colon samples were subsequently divided in two aliquots. One aliquot of each was snap frozen in liquid nitrogen and stored at −80°C for further analyses, the other was fixed in 4% formaldehyde overnight, subsequently transferred in ethanol 70% and paraffin-embedded.

2.3. DNA extraction from fecal samples

Fresh fecal samples of 32 rats (8 replicates for each of the four different groups) were collected at the end of the treatment (after 6 weeks). Total DNA extraction from samples was carried out by using the QIAamp Fast DNA stool kit (Qiagen, Hilden, Germany, catalog n. 51604), according to the manufacturer's instructions [33]. The amount and the quality of the extracted DNA was evaluated using Nanodrop ND-2000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and a high-sensitivity Qubit fluorometer.

2.4. High-throughput sequencing and bioinformatic analysis

Partial 16S rRNA gene sequences were obtained using primer pairs 314F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) targeting the V3-V4 of the 16S rRNA gene region [34,35]. The amplicons' sequencing was performed by Novogene (https://www.novogene.com) using an Illumina MiSeq machine. All sequences were imported in R and analyzed with the DADA2 package, as previously reported [36]. Following the package guidelines, quality plots were performed to check the sequences' quality and post-QC reads were trimmed using the filter and Trim command. Paired-end reads were merged, and exact Amplicon Sequence Variants (ASVs) inferred using the dada algorithm. Chimeric sequences were removed, and prokaryotic taxonomy assigned using the naive Bayesian classifier method QIIME2 [37,38] against the Silva Database v. 138. The obtained sequences are available in the NCBI Sequence Read Archive (SRA) database under the bioproject number PRJNA998626. BioSample accession number for each sequence is included in Supplementary Table S2.

Alpha-diversity was calculated using the observed number of ASV, Simpson diversity index and Shannon diversity index. Beta diversity was measured with the principal coordinate analysis (PCoA) based on Weighted Unifrac distance.

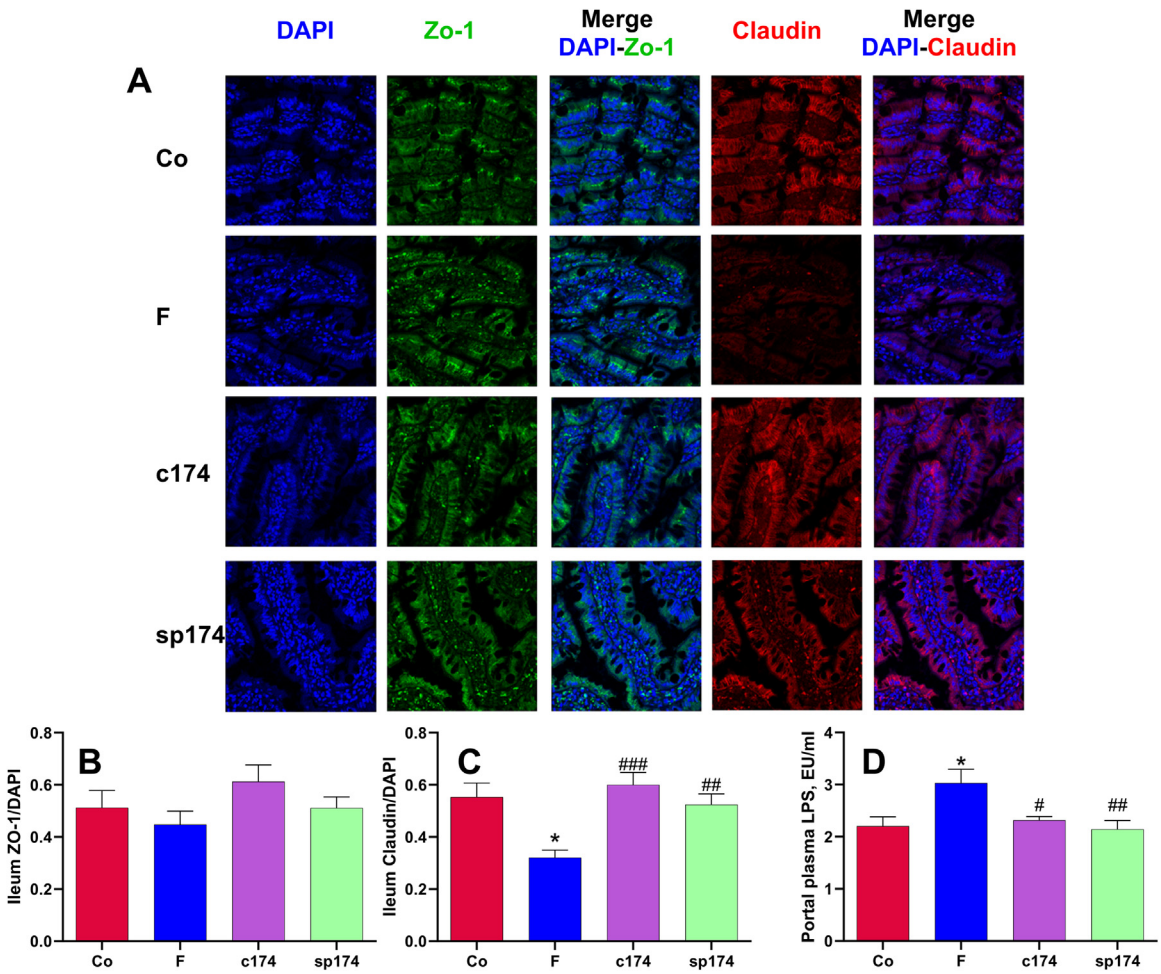

Fig. 1. Gut barrier integrity. ZO-1 (A) and Claudin (B) immunostaining with representative images (C), portal plasma lipopolysaccharide (LPS) (D) in the ileum from rats fed control diet (Co), fructose-rich diet (F), and supplemented with cells (c174) or spores (sp174) of *Shouchella clausii* SF174 for 6 weeks. Values are the means ± SEM of eight different rats. *P<0.05 compared to Co rats; # P<0.05, ## P<0.01, ### P<0.001, compared to F rats (One way ANOVA followed by Tuckey post-test).

The relative abundances were reported as center log ratio (CLR) transformed values. Statistical differences in the genus composition among different groups were tested using the Krukal–Wallace test followed by BH p-value adjustment from the Aldex2 package. The genera that resulted significantly different in the Kruskal-Wallace test underwent a posthoc analysis procedure. Posthoc analysis was performed by means of a set of pairwise comparisons between the fructose treated groups by using the Wilcoxon tests from the Aldex2 R package and correcting for multiple testing with BH [39].

2.5. Mass spectrometry determination of SCFAs and lactate in fecal samples

Lactic acid, butyric acid, propionic acid and acetic acid in rat fecal samples were quantified by liquid chromatography high resolution mass spectrometry (LC-HRMS). Feces (50 mg) were suspended in 300 μL of 75% v/v methanol along with 100 mg of glass beads. Fecal suspensions were mixed, then homogenized through high-speed shaking, two cycles at 30 Hz, 2 min (TissueLyser II, Qiagen, Hilden, Germany). Samples were centrifuged (18000xg, 10 min, 4°C) and 10 μL of fecal supernatants was spiked with 1 μL of carbon labelled internal standard mix including $^{13}C_2$-acetate, $^{13}C_3$-propionate $^{13}C_4$-butyrate (final concentration 0.1 mM for each compound). For the derivatization procedure, 60 μL of 75% v/v methanol, 60 μL of 3-NPH (200 mM) and 10 μL of EDC (120 mM in 6% pyridine) were subsequentially added. Derivatization reaction was stopped with the addition of 10 μL quinic acid (200 mM). Samples were centrifuged at 18000xg for 5 min at 4°C, and supernatants diluted up to 1 mL with 10% v/v methanol, and directly injected (5 μL) without any further dilution. Quantitation of short chain fatty acid (SCFA) hydrazone derivatives was achieved by a U-HPLC system (Ultimate 3000 RS, Thermo Fisher Scientific, Bremen, Germany) interfaced to a linear ion trap hybrid Orbitrap high resolution mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific) equipped with an electrospray ion source (ESI) working in negative ion mode. Mobile phases consisted of water (solvent A) and acetonitrile (solvent B), and the flow rate was 0.2 mL/min. Analytes were eluted with the following gradient of solvent B (minutes/%B): (0/5), (5/5), (12.3/35), (13.3/85), (14/99), (16/99) by using a reversed phase C18 column (Kinetex C18 PS, 100 × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA), thermostated at 40°C. Ion source interface parameters were the following: capillary temperature was 300°C, sheath and auxiliary gases were set at 25 and 15 arbitrary units, respectively. Orbitrap detector scanned the ion in the m/z range 100-400 and the resolution was set at 30000 (FWHM at m/z 200). Analyte profile data in full MS mode were recorded using Xcalibur 2.1 (Thermo Fisher Scientific). Calibration curve was obtained through the internal standard technique in the linearity

range 0.001-1 mM by using the same derivatization procedure detailed above for fecal samples. Analytical performances are detailed in Supplementary Table S3.

2.6. Inflammatory markers analysis in portal plasma, ileum and colon

Portal plasma samples were obtained from the portal blood collected in tubes containing EDTA at the time of sacrifice and subsequently centrifuged for 15 minutes at 1400 x g and subsequently stored at −20°C.

Lypopolisaccharide (LPS) in portal plasma was determined, as previously reported [40], using a protocol based on a Limulus amaebocyte lysate (LAL) extract (ThermoFisher Scientific, Rockford, IL, USA, catalog n. A39552), in accordance with the kit instructions.

In brief, samples were mixed with the LAL reagent, at 37°C. After 20 minutes of incubation, the Chromogenic Substrate solution was added for 6 min at 37°C and absorbance readings were taken on a plate reader at 405 nm.

Concentrations of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were assessed with enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA, catalog n. DY510, catalog n. DY506, catalog n. DY522 respectively), specific for rats, in accordance with the kit instructions, in proteins extracts from proximal ileum and colon.

Intestinal alkaline phosphatase (IAP) activity was measured in proximal ileum and colon. Ileum and colon tissues for the analysis were prepared according to Kaliannan et al. [41] and IAP activity was determined following the reported protocol [42], with the addition of the selective IAP inhibitor phenylalanine (10 mM), in order to subtract the result with phenylalanine from the result without phenylalanine [42]. The specific activity of the enzyme is expressed as picomoles pNPP hydrolyzed/min/μg of protein. A protein assay reagent from Fisher Scientific (Pierce reagent, catalog n. 22660) was used to determine protein concentration in each sample.

2.7. Western blot

Proteins were extracted from colon and ileum by homogenizing frozen tissues (-80°C) in five volumes (w/v) of lysis buffer containing 20 mM Tris-HCl (pH 8), 138 mM NaCl, 2.7 mM KCl, 5% (v/v) glycerol, 1% (v/v) Nonidet P-40, 5 mM EDTA and 5% of protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (all from Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 21952xg for 10 min at 4°C and the supernatants were collected. Aliquots of protein extracts were denatured in a buffer (60.0 mmol/L Tris, pH 6.8, 10% sucrose, 2% SDS, 4% β-mercaptoethanol) and loaded on a 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel. After the run in electrode buffer (50 mmol/L Tris, pH 8.3, 384 mmol/L glycine, 0.1% SDS), the gels were transferred on PVDF membranes at 0.8 mA/cm^2 for 90 min. The membranes were preblocked in blocking buffer (PBS, 3% bovine albumin serum, 0,3% Tween 20) for 1 h and then incubated overnight at 4°C with antibodies against toll-like receptor 4 (TLR4) (Santa Cruz biotechnology, Dallas, TX, USA, catalog n. sc-293072; diluited 1:1000 in blocking buffer), glucose transporter 5 (GLUT-5) (Invitrogen, Carlsbad, CA, USA, catalog n. PA580023; 0.5 μg/mL in blocking buffer), zonula occludens 1 (ZO-1) (Invitrogen, Carlsbad, CA, USA, catalog n. 61-7300; 2μg/mL in blocking solution) and claudin-1 (Invitrogen, Carlsbad, CA, USA, catalog n. 37-4900; 2μg/mL in blocking solution). Membranes were washed three times for 7 min in PBS/0.3% Tween 20, and then incubated for 1 h at room temperature with an antimouse for TLR4 and claudin-1 and antirabbit for GLUT-5 and ZO-1 HRP-conjugated secondary antibody. The membranes were washed as described above, rinsed in distilled water, and incubated at room temperature with a chemiluminescent substrate, Immobilon HRP substrate (Millipore Corporation, Billerica, MA 01821, USA, catalog n. WBKLS0500). Quantitative densitometry of the bands was carried out by Image Lab Software (Biorad). Actin was detected with polyclonal antibody (Sigma-Aldrich, St Louis, MO, USA; catalog n. A2066; diluted 1:1000 in blocking buffer) and used to normalize the TLR4 and GLUT-5 signals.

2.8. Histological analysis

Samples taken from the ileum and the colon were used for histological analysis. The samples were fixed overnight in 4% paraformaldehyde and then dehydrated with different concentrations of ethanol. After dehydration, samples were embedded in paraffin and were subsequently cut into 4-μm thick sections. The prepared tissue sections were stained with hematoxylin and eosin (H&E) and the villi height (VH), crypt depth (CD), and the relative ratio were evaluated. Images were acquired using a Zeiss Primostar 3 at 10X magnification Three random field/section per rat were analyzed and scored blindly using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.9. Immunofluorescence analysis

The ileum and colon tissue section were deparaffinized and rinsed with distilled water for 5 min. Tissue sections were then subjected to antigen retrieval with DAKO solution (catalog n.S1699). The sections were blocked with 2% bovine serum albumin and then incubated overnight at 4°C with antibodies against ZO-1 (Invitrogen, Carlsbad, CA, USA, catalog n. 61-7300; 2μg/mL in blocking solution) and claudin-1 (Invitrogen, Carlsbad, CA, USA, catalog n. 37-4900; 2μg/mL in blocking solution) and subsequently were stained with DAPI (Sigma Aldrich, Saint Louis, MO, USA, catalog n. D9542; diluted 1:500 in PBS). For the analysis, images were captured and visualized using Zeiss Confocal Microscope LSM 700 at 40X magnification, using a drop of immersion oil (Immersoil 518 F, Zeiss). Three random field/section per rat were analyzed and scored blindly using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.10. Oxidative stress and antioxidant enzymes activity determination in ileum and colon

Oxidative stress markers were evaluated in ileum and colon homogenates made in 50 mM phosphate buffer, pH 7.0 (1:50 w/v).

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay as previously described [43].

Superoxide dismutase (SOD) activity was measured by following the decrease in the reduction rate of cytochrome *c* by superoxide radicals in a medium containing 50 mM KH_2PO_4 pH 7.8, 20 mM cytochrome *c*, 0.1 mM xanthine, and 0.01 units of xanthine oxidase, as previously reported [40].

Catalase activity was measured in 50 mM phosphate buffer, pH 7.0 containing 10 mM H_2O_2 and 0.25% Triton X-100 by monitoring the decomposition of H_2O_2 at 240 nm as described previously [44].

Glutathione reductase (GR) activity was measured following the decrease of NADPH absorbance at 340 nm, as described previously [45]. The reaction mixture contained 0.2 M, potassium phosphate buffer, 2 mM EDTA, 2 mM NADPH (in 10 mM Tris-HCl, pH 7), and 20 mM oxidized glutathione. The activity was calculated using the

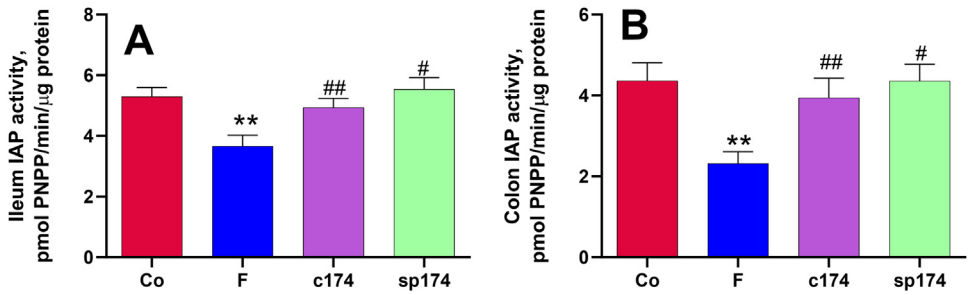

Fig. 2. Intestinal alkaline phosphatase (IAP) activity in the ileum and colon. IAP activity (A, B) in ileum and colon from rats fed control diet (Co), fructose-rich diet (F), and supplemented with cells (c174) or spores (sp174) of *Shouchella clausii* SF174 for 6 weeks. Values are the means ± SEM of eight different rats. $^{**}P<.01$ compared to Co rats; # $P<.05$, ## $P<.01$, compared to F rats (One way ANOVA followed by Tuckey post-test).

NADPH molar extinction coefficient, 6.22×10^{-5}, considering that one unit of GR is defined the amount of enzyme that catalyses the reduction of 1 μmol of NADPH per minute. The specific activity is expressed in U per g of tissue.

NADPH oxidase activity was evaluated according to a modification of the method of Bettaieb et al. [46]. Briefly, tissues (1:10 w/v) were homogenized in ice-cold Krebs buffer and then centrifuged at 800xg, at 4°C for 10 min. The supernatant was collected and then centrifuged at 30000xg for 2h at 4°C. The pellet (membrane fraction) was resuspended in Krebs buffer and protein concentration measured. Aliquots containing 100 μg of protein were added to Krebs buffer containing NADPH (500 μM). The change in absorbance at 340 nm was followed for 10 min at 30 s intervals.

2.11. Enzymatic colorimetric assays

Fructose levels in ileum and colon were assessed using colorimetric enzymatic from Sigma Aldrich (St. Louis, MO, USA, catalog n. FA20). Fructose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Fructose 6-phosphate is converted to glucose 6-phosphate by phosphoglucose isomerase (PGI). Glucose 6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD) in the reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The resulting increase in absorbance at 340 nm is directly proportional to the concentration of fructose in the sample. The protocol includes a blank for the PGI, a blank for the sample, a blank for the assay reagent (Glucose Assay Reagent, GAR) and the sample.

The plate was incubated for 15 minutes at room temperature, after which the absorbance was measured at 340 nm. The mg of fructose contained in the sample were obtained using a formula that take into account the difference in absorbance (ΔA), the total and the sample volume incubated, the fructose molecular weight, and the NADH molar absorption coefficient (ε), as manufacturer instructions.

Uric acid levels in ileum and colon were assessed using colorimetric enzymatic from GS Diagnostics SRL (Guidonia Montecelio, Rome, Italy, catalog n. 4059) is converted by the enzyme uricase into allantoin, with the formation of hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with the chromogenic substrate constituted by 4-aminoantipyrine leading to the formation of a colored compound whose concentration is proportional to the concentration of uric acid present in the sample. The plate is read at a wavelength of 510 nm. The absorbance values obtained for the sample and the standard are then used to determine the uric acid concentration (mg/dl).

2.12. Determination of counts of SF174

SF174 cells and spores were counted as previously reported [47]. Fecal samples (approx. 1 g) from each animal of each group were suspended in the minimal volume of sterile PBS that allowed adequate suspension of solid matter by vigorous vortexing. Each suspension was divided in two halves and each transferred in a sterile tube. One tube for each sample was ethanol treated: 1 volume of homogenised sample (approx. 100 microL) was mixed with an equal volume of absolute alcohol and incubated for 1 h at room temperature. Samples were then immediately serially diluted and plated out on solid Difco Sporulation-inducing (DS) medium supplemented with [8 μg/ml] streptomycin, [4 μg/ml] clindamycin, [4 μg/ml] erythromycin and [8 μg/ml] chloramphenicol to allow the growth of SF174 cells, resistant to all four antibiotics [29]. Plates were incubated for 2-4 days at 37°C and about 20 randomly selected colonies for each plate observed under the light microscope to evaluate the morphology of cells, sporangia and spores.

2.13. Statistical analysis

The animal physiological data are reported as mean values ± SEM. GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was utilized to check the normal distribution of the raw data and to perform One way ANOVA. In all analyses, a $P<.05$ (probability<5%) was considered statistically significant. The analysis of the fecal microbial composition was performed with SPSS software v. 25 (www.ibm.com/software/it/analytics/spss/). The Analysis of Variance (ANOVA) was used to compare differential abundance of bacterial genera and differences with a P-value $<.05$ were considered significant.

3. Results

3.1. Tight junction protein content and portal plasma endotoxemia

We here evaluated whether cells or spores of the SF174 strain counteracted the fructose-induced damages at the level of the gut barrier. To this aim we analyzed by immunofluorescence confocal microscopy and by Western Blot the levels of the tight junction proteins ZO-1 and claudin in the ileum and colon. Representative images of ileum immunofluorescence are shown in Fig. 1A, the quantification a representative western blot images are shown in Supplementary Fig. SA and B, for claudin and ZO-1 respectively. In the colon, neither protein was affected by the fructose-rich diet (Supplementary Fig. S3E, F and Supplementary Fig. S4A, B). In the ileum, while ZO-1 was not affected (Fig. 1B), the claudin-1 levels were reduced by fructose and such reduction was avoided by the treatment with both cells and spores of SF174 (Fig. 1). In

agreement with the effects on the claudin-1 levels, portal LPS was higher in F than in Co rats and the effect was prevented by both cells and spores of SF174 (Fig. 1D), thus suggesting that SF174 cells and spores exert a significant protective effect against the derangement of gut barrier integrity, thus avoiding endotoxemia. These results were confirmed by the histopathological evaluation of both ileum and colon section, that showed in fructose fed rats shortened villi and longer crypt with a subsequent significant decrease in VH/CD ratio only in the ileum but not in the colon. These alterations of the intestinal architecture were avoided in c174 and sp174 rats, strengthening the efficiency of *S. clausii* SF174 administration in protecting the intestinal barrier (Supplementary Fig. S5).

3.2. Modulation of IAP activity and inflammatory response in the ileum and colon

In our experimental model, IAP activity was downregulated by fructose-rich diet both in ileum (Fig. 2A) and colon (Fig. 2B), and the probiotic administration counteracted this decrease. In addition, the proinflammatory effect of LPS in fructose-fed rats was potentiated by the increase in the LPS receptor TLR4 in the ileum that was prevented by administration of cells or spores of SF174 (Fig. 3A). Ileal cells also displayed significantly higher content of the proinflammatory cytokines TNF-α (Fig. 3B) and IL-6 (Fig. 3C), that was avoided when rats were treated with either cells or spores of the SF174. No effect was found on the anti-inflammatory cytokine IL-10 (Fig. 3D). Differently from ileum, all the above changes were not detected in the colon (Fig. 3E, F, G, H).

3.3. Oxidative balance in ileum and colon

In ileum of fructose fed rats, a condition of oxidative damage was evidenced by the increased TBARS levels (Fig. 4A), and a decrease in the antioxidant enzyme catalase and GR (Fig. 4B, D), that vanished the protective counterregulatory increase in SOD activity (Fig. 4C). c174 and sp174 treatments reversed all the above changes except that the increased SOD in sp174 (Fig. 4).

The same pattern of oxidative unbalance was found in the colon of F rats, and also in this district both spores and cells of *S. clausii* SF174 were completely effective in preventing all the above changes (Fig. 4E, F, G, H).

3.4. Intestinal microbiota composition

We analyzed the gut microbiota of our experimental groups by a 16S sequencing approach. Analysis of the alpha-diversity indicated that the spores-treated group was characterized by a higher diversity with a more equal distribution (number of observed features and Shannon index, Fig. 5A, B) than all other groups. In line with the observed higher alpha-diversity, the spore-treated group also showed the higher number of unique ASVs (2540 specific taxa) (Supplementary Fig. S6).

The beta-diversity analysis, performed to compare the microbial composition between the samples, revealed that the gut microbiota of animals of the control, fructose, and spore-supplemented groups (Fig. 5C) formed independent clusters. On the contrary, the gut microbiota of cell-supplemented animals did not form a clear cluster (Fig. 5C), and each individual of that group was scattered and overlapped with clusters of the other three groups.

The effect of the probiotic treatments on the microbiota composition was evaluated at the genus level using a compositional approach [48]. In particular, a CLR transformation of the abundance values was applied, and the relative abundances compared by performing a univariate statistical test using the ALDEx2 tool. The significant differences between the probiotics treated groups (c174 and sp174) with respect to the fructose treated group (F) were evaluated using Wilcoxon tests [49]. The analyses revealed that 23 genera were altered by the treatment with SF174 spores (Fig. 6A), with a reduction of the relative abundance observed in the sp174 group with respect to the F group, while only 2 genera (*Streptomyces* and *Ruminococcus torque*) resulted increased in the sp174 group (Fig. 6A). Only five genera were altered by the treatment with both SF174 cells and SF174 spores (Fig. 6A, B), while no significant variation was found between control and fructose-fed groups.

A similar number of 16S reads relative to the *Bacillus* genus was found in the four experimental groups, independently from the supplementation with SF174 cells or spores (Supplementary Table S2). This result could be due to the low efficiency of DNA extraction from bacterial spores that often causes the under-estimation of endospore-forming bacteria in environmental diversity surveys [50,51]. To evaluate the presence of SF174, the same fecal samples used to extract total DNA for 16S sequencing were analyzed for the presence of live SF174 cells or spores by CFU determination, taking advantage of the antibiotic resistances of SF174 [33]. As shown in Table 1, SF174 was not detected in animals of the groups not treated with the probiotics while an average of 42.6×10^2 and 3.3×10^2 CFU of SF174 per gram of faecal sample were recovered from animals treated with spores or cells, respectively. Similar CFUs were obtained when ethanol was used to discriminate between ethanol-resistant spores and ethanol-sensitive vegetative cells present in the sample [48], indicating that all the counted CFUs were due to spores present in the faecal samples (Table 1).

Table 1
Colony Forming Units (CFU) from faecal samples*

Group	Untreated samples	Ethanol-treated samples
Co	-	-
F	-	-
c174	3.3±2.1	3.6±1.7
Sp174	42.6±1.0	43.3±1.4

* Value are x10^2 CFU per gram.

3.5. Short chain fatty acids, lactate and colon NADPH oxidase

SCFA, such as acetate, propionate, butyrate are main bacterial metabolites produced in the colon following the fermentation of dietary fibers and resistant starches [48]. We here found increased levels of acetate, propionate, butyrate and lactate in fecal samples of fructose fed rats compared to the controls (Fig. 7). Interestingly, the administration of cells and spores of *S. clausii* SF174 did not affect acetate and butyrate (Fig. 7A, B), but significantly decreased lactate levels, while increased propionate levels with respect to rats fed with fructose alone (Fig. 7C, D). A significant increase in NADPH oxidase activity was found in F rats, but not in c174 and sp174 rats (Fig. 7E), in line with the previous reported stimulation of colonic NADPH oxidase by excess of lactate [52].

3.6. Fructose, uric acid and GLUT-5 content in ileum and colon

Fructose, the fructose transporter GLUT-5 and acid uric were increased in the ileum (Fig. 8A, B, C) but not in the colon (Fig. 8D, E, F) of animals of the F group, as shown in Fig. 8. In the ileum, the effects were totally reversed by both cells or spores of SF174 (Fig. 8 A, B, C). The increased uptake of fructose in the ileum of F rats was confirmed by increased portal plasma fructose, while no variation was evident in the animals treated with cells or spores (Fig. 8G).

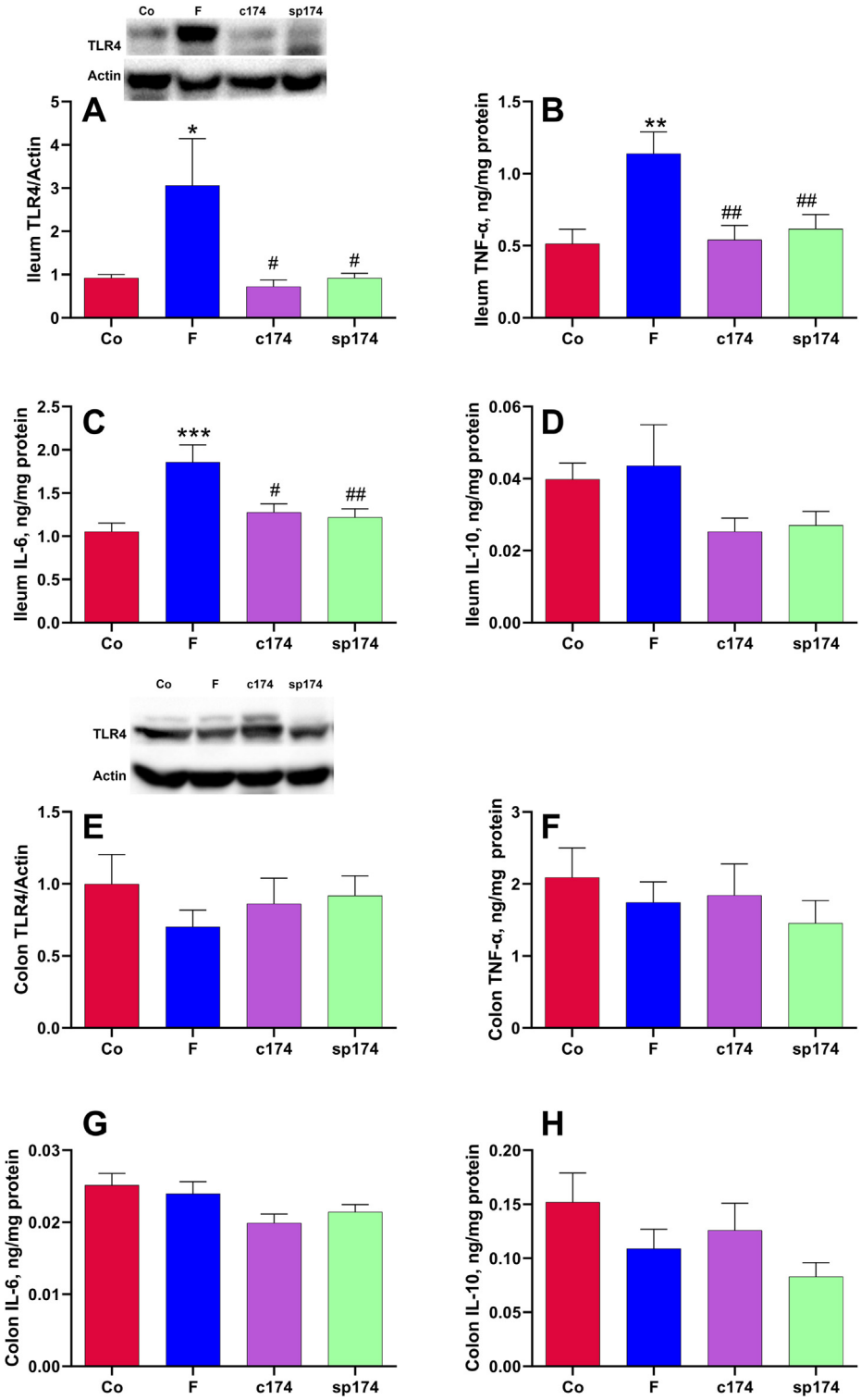

Fig. 3. Inflammatory profile in ileum and colon. TLR4 with representative Western Blot (A, E), Tumor necrosis factor (TNF)-α (B-F), Interleukin-6 (IL-6) (C-G), Interleukin-10 (IL-10) (D-H) in ileum and colon from rats fed control diet (Co), fructose-rich diet (F), and supplemented with cells (c174) or spores (sp174) of *Shouchella clausii* SF174 for 6 weeks. Values are the means ± SEM of eight different rats. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ compared to Co rats; #$P<.05$, ##$P<.01$compared to F rats (One way ANOVA followed by Tuckey post-test).

In addition, the analysis of the SF174 genome revealed the presence of homologs of several genes involved in fructose transport and catabolism (Table 2), and SF174 cells were shown to be able to grow on fructose as the only carbon source (Fig. 9).

4. Discussion

Increased fructose intake determines its higher absorption in the ileum by its specific transporter, namely GLUT-5 [53], and as a

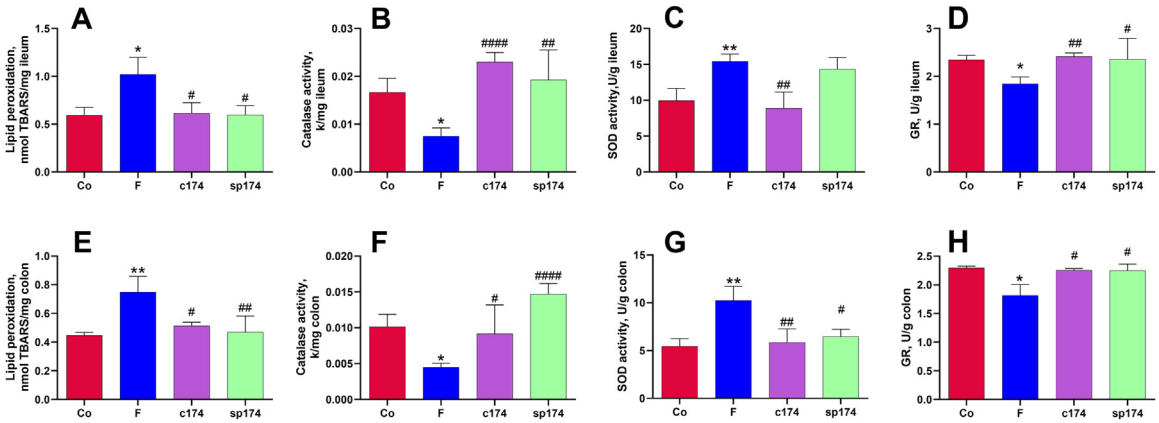

Fig. 4. Oxidative balance in the ileum and colon. Lipid peroxidation (A, E), catalase activity (B, F), superoxide dismutase (SOD) activity (C, G) and glutathione reductase (GR) activity (D, H) in ileum and colon from rats fed control diet (Co), fructose-rich diet (F), and supplemented with cells (c174) or spores (sp174) of *Shouchella clausii* SF174 for 6 weeks. Values are the means ± SEM of eight different rats. $^{*}P<.05$, $^{**}P<.01$ compared to Co rats; #$P<.05$, ##$P<.01$, #### $P<.0001$ compared to F rats (One way ANOVA followed by Tuckey post-test).

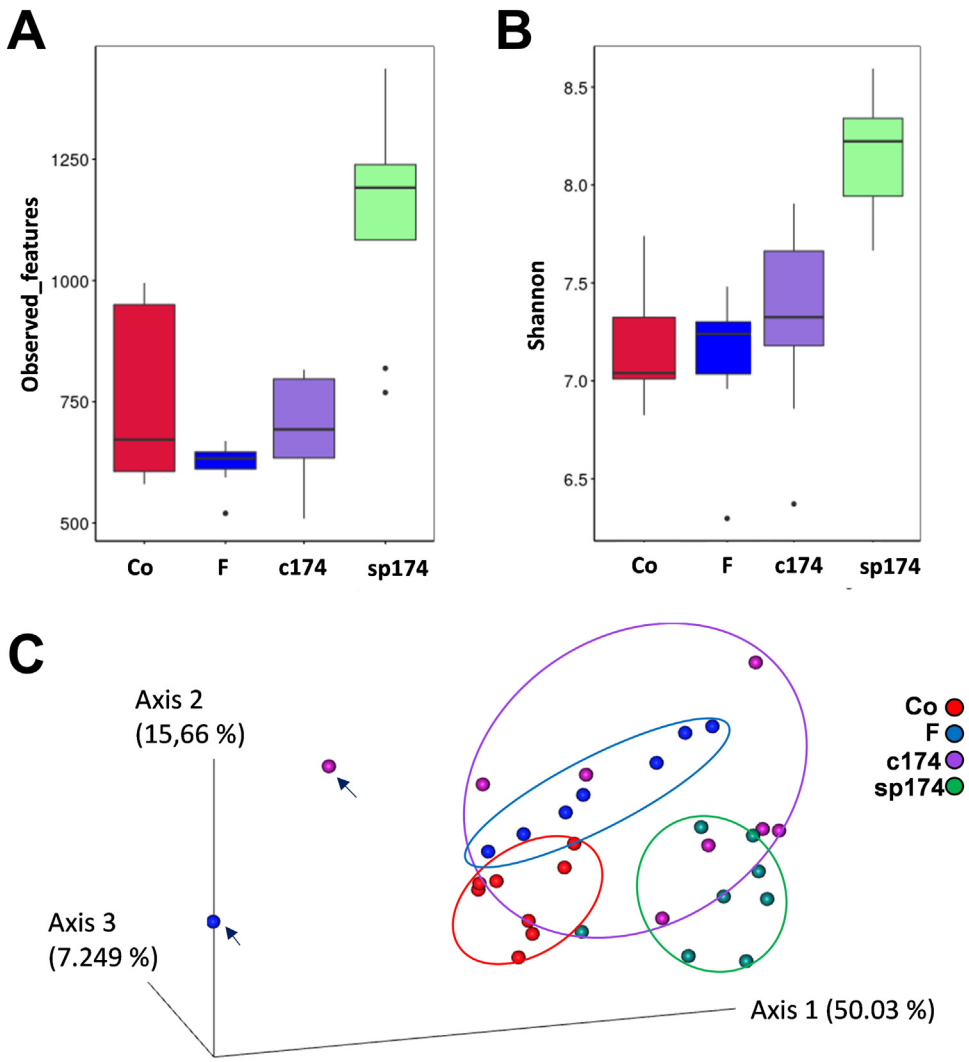

Fig. 5. Alpha diversity analysis and beta diversity analysis. The microbial diversity was analyzed by using the Observed-ASV (A) and the Shannon (B) indeces. The Principal Coordinate Analysis (PCoA) plot (C) was generated using weighted UniFrac distance matrix. The four experimental groups are marked by different colours as indicated. The circles were used to identify rats fed control diet (Co), fructose-rich diet (F) and supplemented with cells (c174) or spores (sp174) of *Shouchella clausii* SF174 for 6 weeks. The line inside each box represents the median value. Outliers are shown as dots. The arrows indicate 2 individuals outside of the clusters.

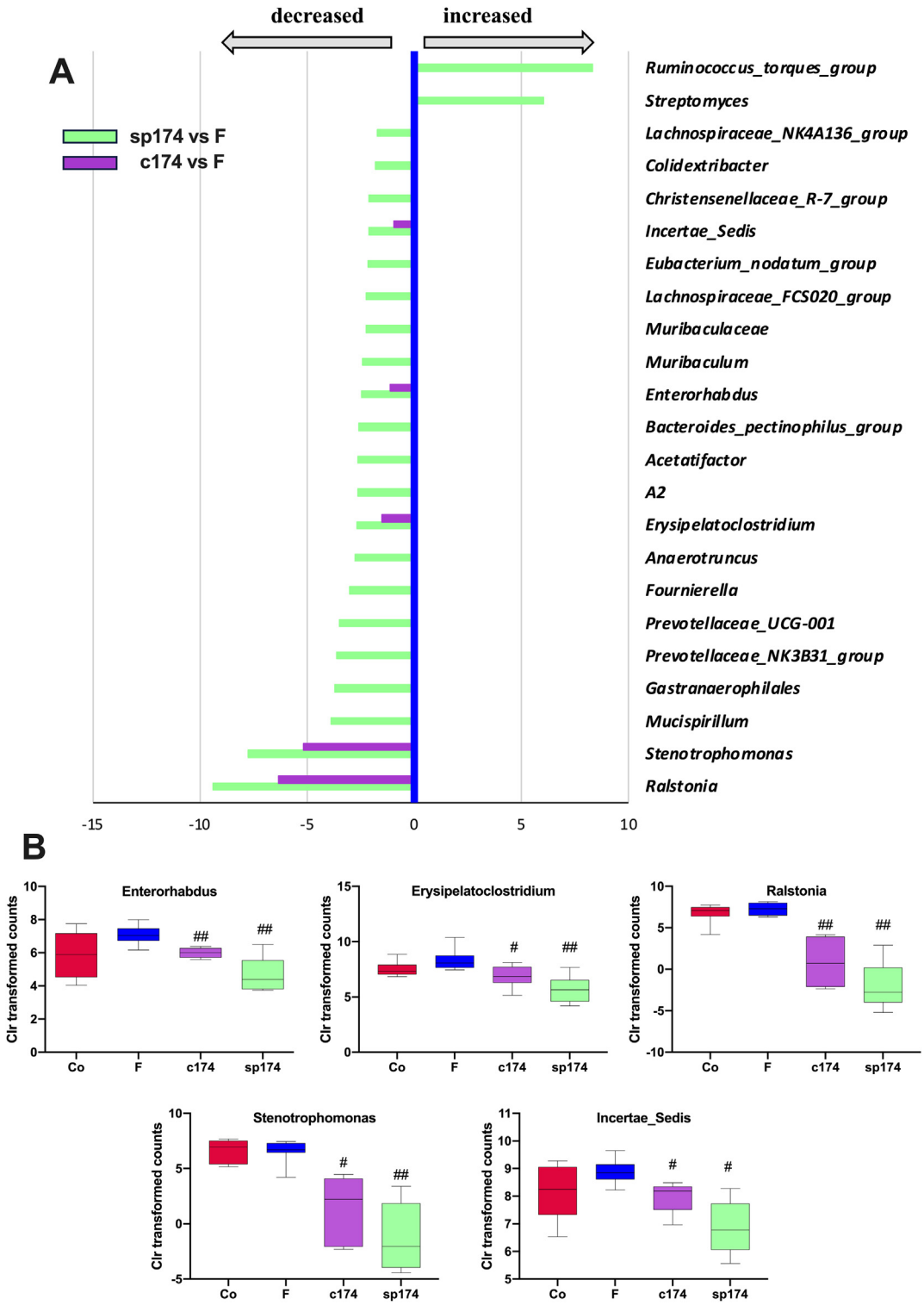

Fig. 6. Statistically significant alterations of the gut microbiota. (A) Genera with a statistically significant variation ($P<.05$) in the animals supplemented with SF174 spores (sp174, green) or SF174 cells (c174, purple) with respect to fructose fed animals. B) Differential representation of the five genera affected by both spores and cells in the Co, F, sp174 and c174 groups. Significance was assessed using a KW test on Centered-Log Ratio (Clr) transformed relative abundance values. The KW P values are indicated in the plots.

consequence, the ileal cells display a significantly higher fructose. Fructose is then immediately converted into fructose-1-phosphate, leading to ATP depletion [54,55,56] and increasing production of uric acid, its main metabolic byproduct [57,58], which in turn contributes to the development of oxidative imbalance [59]. In agreement, after 6 weeks of fructose feeding, we report here increased uric acid levels and oxidative stress, notwithstanding the different effect on antioxidant enzymes, namely catalase and GR, whose ac-

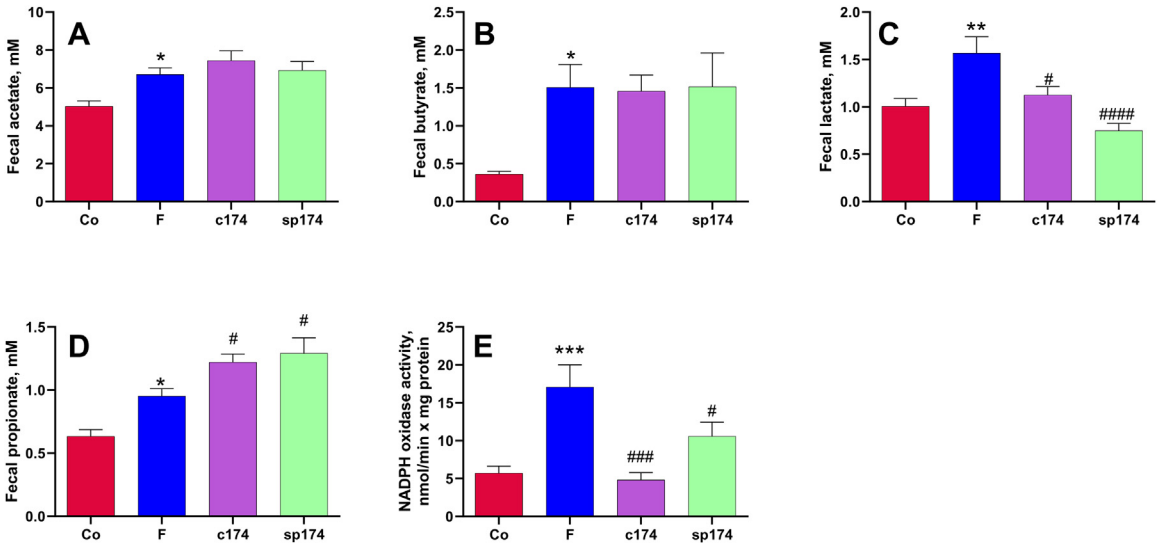

Fig. 7. Short chain fatty acids and lactate. Quantification of acetate (A), butyrate (B), lactate (C) and propionate (D) in faecal samples and colonic NADPH oxidase activity (E) in rats fed control diet (Co), fructose-rich diet (F), and supplemented with cells (c174) or spores (sp174) of *Shouchella clausii* SF174 for 6 weeks. Values are the means ± SEM of eight different rats. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ compared to Co rats; $\#P<.05$, $\#\#\#P<.001$, $\#\#\#\#P<.0001$ compared to F rats (One way ANOVA followed by Tuckey post-test).

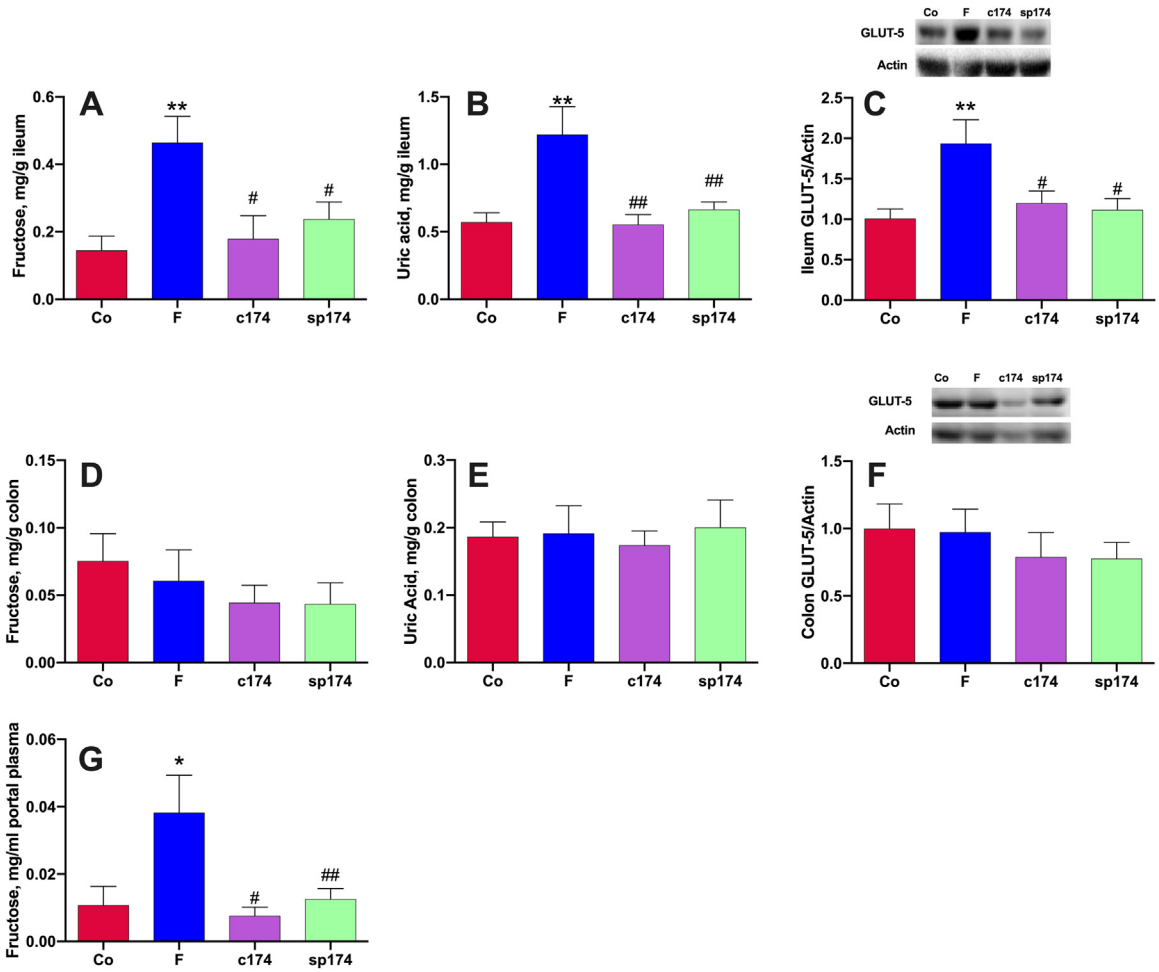

Fig. 8. Content of fructose, uric acid and GLUT-5 in the ileum and colon. Content of fructose (A, D), uric acid (B, E) and GLUT-5 with representative Western Blot (C, F) in ileum and colon, together with portal plasma fructose (G) from rats fed control diet (Co), fructose-rich diet (F), and supplemented with cells (c174) or spores (sp174) of *Shouchella calusii* SF174 for 6 weeks. Values are the means ± SEM of eight different rats. $^{*}P<.05$, $^{**}P<.01$ compared to Co rats; $\#P<.05$, $\#\#P<.01$ compared to F rats (One way ANOVA followed by Tuckey post-test).

Table 2
Genes of SF174 coding for homolog of proteins involved in the fructose transport and catabolism in the reference strain *S. clausii* KSM-K16.

Locus_tag of SF174	Product	Protein ID of *S. clausii* KSM-K16	Protein identity (%)
SF174_3234	fructokinase	WP_142237578.1	100
SF174_3109	PTS system, fructose-specific IIC component	WP_011246122.1	99.37
SF174_3110	1-phosphofructokinase	WP_011246121.1	99.67
SF174_3111	Transcriptional regulator of fructose utilization, DeoR family	WP_011246120.1	100
SF174_0942	fructose-bisphosphate aldolase	WP_011245306.1	100
SF174_0943	PTS fructose transporter subunit IIA	WP_011245307.1	100
SF174_0944	PTS fructose transporter subunit IIB	WP_011245308.1	100
SF174_0945	PTS fructose specific enzyme IIC	WP_011245309.1	100
SF174_1376	PTS fructose transporter subunit IIC	WP_011245714.1	99.84
SF174_3260	1-phosphofructokinase	WP_011248040.1	100
SF174_3261	PTS fructose transporter subunit IIC	WP_011248039.1	100
SF174_0111	6-phosphofructokinase	WP_011247562.1	99.69

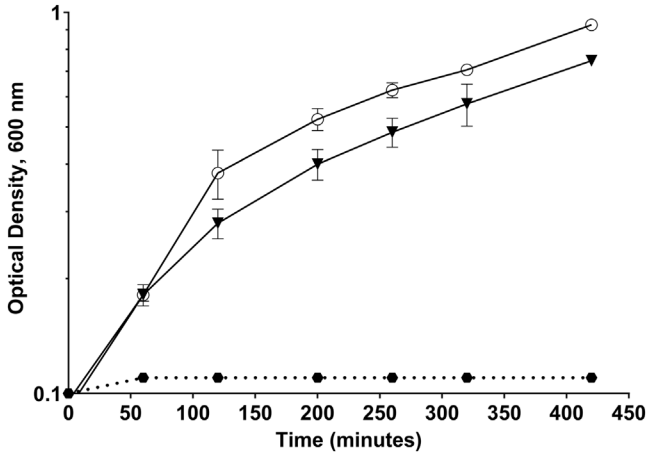

Fig. 9. Growth curves. Optical density of SF174 cultures grown in modified S7 minimal medium (Methods) not supplemented with a carbon source (closed exagons) or supplemented with 1% glucose (open circles) or 1% fructose (close triangles). Data are the average of three independent experiments.

tivity was found decreased, while an increased activity of SOD was measured, probably as compensatory response, already evidenced in the colon in high fructose fed rats even after only 3 weeks of diet [40]. This counterregulatory response has been reported by other authors as well, also at the level of other organs, such as the liver [60–62]. Another major effect we observed is the fructose-induced reduction at the ileum level in the amount of claudin-1, a tight junction protein, which determines an increase in the intestinal barrier permeability and translocation of LPS, a bacterial endotoxin, from the intestine to the portal plasma, a condition known as endotoxemia [63]. LPS has a strong inflammatory effect due to its ability to bind the TLR4 receptor and inducing the release of proinflammatory cytokines [64]. In physiological conditions, LPS accumulation is prevented also by the anti-inflammatory action of IAP, an endogenous enzyme expressed by the intestinal epithelium [65], able to detoxify the LPS by removing the phosphate from its lipid A moiety [66], thus preventing its recognition by TLR4, and consequent cell activation. In our experimental model, IAP activity was downregulated by fructose-rich diet and this induces an inflammatory status in the ileum, here shown by the increased levels of proinflammatory cytokines, TNF-α and IL-6.

Surprisingly, at the level of the colon, a completely different situation was found. Indeed, it seems that fructose does not reach the colon at all, as no changes in GLUT-5, fructose or uric acid levels, and in turn no alterations in claudin-1 and no sign of inflammation were detected. On the other hand, oxidative unbalance was found in fructose fed rats at the level of the colon as well. This can be explained by the lactate overproduction observed in fructose fed rats. High levels of lactate in the colon are able to trigger the generation of reactive oxygen species (ROS) through the upregulation of the colonic enzyme NADPH oxidase [52], suggesting that the fructose-induced oxidative stress in the colon is due to lactate accumulation. Such suggestion is supported by the increased levels of NADPH oxidase that we observed only in fructose fed rats. Lactate can be converted into propionate by members of the *Lachnospiraceae* family [67], whose abundance is increased by both cells and spores of *S. clausii* in our experimental system, thus suggesting that the oxidative stress reduction in the colon of probiotic-supplemented rats depends on lactate clearance. In addition, in our experimental model, the concentration of butyrate, acetate and propionate, were all increased by fructose, in agreement with previous studies showing that higher levels of SCFAs are induced by fructose-rich diet [68,69,70] and are associated with diet-induced NAFLD and obesity [71–74] thus suggesting possible deleterious effects, perhaps depending on SCFA profile and concentration. In fact, SCFA in certain concentrations have also been described to weaken intestinal barrier function and to favour endotoxemia [75].

It is interesting to notice how the administration of both spores and vegetative cells of *Shouchella clausii* SF174, a potential probiotic strain previously isolated from an ileal biopsies of a healthy human volunteer [28], prevented all the above-mentioned fructose induced alterations in the ileum and the oxidative unbalance in the colon, exception made for butyrate, acetate, propionate and lactate levels that were only partly affected by the probiotics, with lactate decreased and propionate increased by both cells and spores of SF174.

Our hypothesis is that cells and germination-derived cells of *S. clausii* have the ability to metabolize fructose at the ileal level, avoiding (or reducing) its uptake by epithelial cells, the consequent uric acid formation and the establishment of oxidative unbalance. This, in turn, would avoid the fructose-induced claudin-1 decrease, the consequent endotoxemia and the inflammatory response, and at the same time would explain the absence of fructose effect on the colon in probiotics' receiver animals, since only a limited amount of fructose would reach the colon. This is confirmed by the fact that both c174 and sp174 rats show all these parameters in a range comparable to the control rat. The support to our hypothesis comes from the ileal origin of SF174 [28] that suggests that it could be specifically adapted to colonize this intestinal site. In addition, we observed that SF174 cells grow *in vitro* on fruc-

tose as the only carbon source and that SF174 genome [32] contains a SF174_3234 gene, 99.66% homologous to the experimentally studied BAD65759.1 gene of the reference strain *B. clausii* KSM-K16 (taxid:66692), and coding for the enzyme fructokinase (EC 2.7.1.4), the first enzyme involved in fructose catabolism.

It is noteworthy that cells and spores of strain SF174 showed similar beneficial effects. Most studies on *Bacillus* probiotics have, so far, focused on the use of spores and all commercial probiotics based on spore-formers contain spores. This is due to the high stability of spores that can safely cross the gastric barrier and ensure an extremely long shelf-life to the commercial products [76,77]. Part of the ingested spores germinates in the small intestine, and temporarily colonize that niche [13], implying that when spores are ingested both spores and germination-derived cells are present at the same time in the small intestine, leaving unanswered the question on which cell form (vegetative cells or quiescent spores) is responsible of the main probiotic effects. Since only cells are present in the small intestine when vegetative cells are ingested [14], the observation that cell and spore treatments have the same physiological effects points to vegetative cells as the main actors of the effects observed in the small intestine.

Finally, we also focused our attention on the study of the gut microbiota, since fructose diet is known to considerably alter it even after a short-term treatment [45] and that probiotic administration could influence its composition. In agreement, our data showed that the fructose-rich diet modified the gut microbiota and that both probiotic treatments partially affected the gut microbiota although in a different way, with 18 genera altered only by spores (16 whose abundance was decreased and 2 increased) and 5 genera similarly decreased by spores and cells, suggesting that spores had a stronger effect than cells on the gut microbiota. This conclusion is not surprising since spores are known to directly affect the composition of the microbiota [21,22]. When spores are orally ingested, some germinate in the small intestine and re-sporulate in the colon while others directly reach the found in fecal samples of animals of the spore- and cell-treated groups (Table 1). However, since the same physiological effects were observed by both probiotic treatments, a possible role of the modified microbiota on the observed physiological effects could only be due to the five taxa decreased by probiotic treatment, of which *Ralstonia* has been previously associated with pathological conditions such as obesity, insulin resistance [78] and ulcerative colitis [79] while *Stenotrophomonas* and *Erysipelatoclostridium* are ubiquitous environmental bacteria, considered as emerging opportunistic pathogens [80], known to produce lactate from carbohydrates metabolism [81], suggesting that the reduced abundance of this taxon in both probiotic-treated groups could explain the decreased levels of lactate found in the colon of these animals.

Our results point to the conclusion that the alteration of the gut microbial composition has a minor effect on the observed probiotic activity of spores and cells of SF174, although it cannot be excluded that the five genera similarly modified by the spore and cell treatment had a role.

In conclusion, our present results put in evidence the beneficial role of *Shouchella clausii* SF174 in the context of high levels of dietary intake of fructose. We hypothesize that, at a mechanistic level, vegetative cells and germination-derived cells metabolise part of the ingested fructose at the ileum level and this on one side avoids its absorption in situ, and the downstream negative consequences of its uptake and metabolism by ileal cells, and on the other side, prevents its arrival in the colon, whose health is affected only by lactate overproduction. These results thus open the way to future studies on how gut microbiota manipulation, including probiotic administration can, in a strain-dependent way, transform host-microbe interactions at defined locations, not only in the colon but also in the small intestine as suggested by recent animal studies and human clinical trials [82,83].

Data deposition

The sequences reported in this paper have been deposited in the NCBI Sequence Read Archive (SRA) database under the bioproject number PRJNA998626.

Data availability statement

The data that support the findings of this study are available from the corresponding author, E.R., upon reasonable request.

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Declarations of competing interests

ER acts as a consultant for Gruppo Savio (Italy) that has the rights for the commercialization of strain SF174. Gruppo Savio (Italy) had no role in the design of the study, in the collection, analyses or interpretation of data or in the writing of the manuscript.

CRediT authorship contribution statement

Anella Saggese: Methodology, Conceptualization. **Valentina Barrella:** Methodology, Conceptualization. **Angela Di Porzio:** Methodology. **Antonio Dario Troise:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Andrea Scaloni:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Luisa Cigliano:** Writing – review & editing, Methodology, Investigation, Data curation. **Giovanni Scala:** Methodology, Investigation, Data curation. **Loredana Baccigalupi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Data curation, Conceptualization. **Susanna Iossa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Ezio Ricca:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Data curation, Conceptualization. **Arianna Mazzoli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2024.109706.

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