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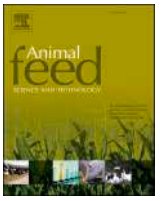

Chestnut extracts decrease the in-vitro digestibility and polyphenol bioavailability of soy-based nutrients but protect the epithelial barrier function of pig jejunum segments after digestion

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ABSTRACT

Polyphenols are known to interact with protein fractions of feed ingredients in the digestive tract thereby affecting the digestibility. These effects may differ depending on the chemical composition of the protein fractions. Thus, the objective of the study was to study the impact of a tannin-rich chestnut extract (CHE) on the in-vitro digestibility (IVD) of soy protein isolate (SPI), screw-pressed soybean (SPS) and solvent-extracted soybean (SES).

In addition, CHE-derived metabolites were tested for their effects on intestinal epithelial integrity. The SPI, SPS, and SES were digested in vitro without (SPI-, SPS-, SES-) or with CHE (SPI+, SPS+, SES+) or as a control (C) solely CHE. The metabolites and the polyphenols content after digestion of the soy-based products with or without CHE were quantified. The trans-epithelial resistance (TEER) was monitored in porcine jejuna in the presence of CHE-derived metabolites at three dilutions (1:4, 1:8, and 1:16 v/v) or in their absence. The claudin-1 (CLDN1), occludin (OCLN) and zonula occludens-1 (ZO1) tight junctions-protein expressions were determined. Finally, the effects of CHE-metabolites against indomethacin (INDO)-induced decrease of TEER were tested *ex vivo*. The CHE decreased the IVD ($P < 0.05$) and decreased the

Abbreviations: Arg, arginine; CD, compound discover; CHEs, chestnut extracts; CLDN1, claudin-1; CP, crude protein; CPD, crude protein digestibility; CTRL, control; DM, dry matter; FS, full scan; GAE, gallic acid equivalent; HT, hydrolysable tannins; INDO, indomethacin; IVCPD, in-vitro crude protein digestibility; IVD, in-vitro digestibility; IVDMD, in-vitro dry matter digestibility; KRB, Kreb's Ringier buffers; OCLN, occludin; OMD, organic matter digestibility; SES, solvent-extracted soybean; SPI, soy protein isolate; SPS, screw-pressed soybean; TEER, trans-epithelial resistance; TJ, tight junction; ZO1, zonula occludens-1.

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detectable polyphenol content ($P < 0.05$) after digestion of CHE with SPI, SPS, or SES. The SPI and SPI+CHE were the samples with the highest content of metabolites generated after the digestion, while CHE presented the lowest content of metabolites. No detrimental effects on TEER were observed. The 1:8 dilution increased ($P < 0.05$) both ZO1 and OCLN protein expression by up to + 60% compared to the control, while the dilution 1:16 increased ZO1 expression by + 100% without affecting OCLN expression. The 1:16 dilution also protected the jejunum from the effects induced by INDO. These results suggest that the anti-nutritional effects exerted by CHE on soy-based meals IVD may depend on the meal's chemical composition. Moreover, we showed that CHE polyphenols exert protective effects on the intestinal epithelium integrity ex vivo at low concentrations.

1. Introduction

Several studies have reported that the use of chestnut extracts (CHEs) as feed ingredients affect different traits in both monogastrics and ruminants, such as gut health and microbial ecosystem (Selje et al., 2007; Tretola et al., 2019; Girard and Bee, 2020). Chestnut (Castanea sativa) is considered a rich source of polyphenols, as they consist of approximately 600 g/kg, as fed glucosidic hydrolyzable tannins (HT) (Aires et al., 2016). The polymers of HT cannot be absorbed in their native form and hydrolysis of linkages by intestinal enzymes and colonic microbiota occurs until they are gradually metabolized to urolithins (Girard and Bee, 2020). Studies have demonstrated that a small amount of CHE-derived metabolites might be intestinally absorbed, exerting antioxidant (Reggi et al., 2020) and cytomodulatory (Cires et al., 2017) activities. Metabolites like punicalagin (Biolley et al., 2019) and gallic acid (Tretola et al., 2021) have also been found to affect the intestinal absorption of specific classes of amino acids. The interaction of HTs from CHE with nutrients, however, can lead to a reduced digestion rate and absorption of proteins, sugars, and lipids (Girard and Bee, 2020). Despite these anti-nutritional effects, the interest in the use of HTs as functional compounds is still high. However, little is yet known about the effects of CHEs on the digestibility of nutrients containing different levels of proteins and lipids, as well as how the CHE-derived polyphenols could affect the intestinal epithelial cell integrity in pigs. To test these interactions in the current study, four in-vitro experimental approaches were used. INFOGEST, a harmonized in-vitro digestion model, was used to mimic the complex and dynamic processes in gastric and small intestinal nutrient digestion (Egger et al., 2019). A second in-vitro digestion approach was used to evaluate the impact of CHE addition on in-vitro organic matter digestibility (OMD) and in-vitro crude protein digestibility (CPD) in three different soy-based products. To test the toxicity of the INFOGEST-digested products and fluids, an in-vitro cell culture model was used to mimic the functional and morphological intestinal organization. In addition, an ex-vivo model using the small intestinal tissue of pigs was used to study the transport and the influence on intestinal integrity of CHE-derived metabolites. The latter, evaluated by protein expression of the tight junction (TJ) proteins, is essential to regulating intestinal permeability and to maintaining the right polarisation of the intestinal epithelial cells, thereby functioning as a barrier against pathogens and other potentially harmful agents (Amasheh et al., 2008). An improved understanding regarding the regulation of intestinal TJs by CHE is of key importance to better estimate the potential and risks associated with the use of HT in feed ingredients. To our knowledge, this is the first time that the harmonized in vitro digestion is coupled with the perfusion chamber system to investigate the interaction between CHE and other nutrients and to directly investigate the effects of the obtained phenolic metabolites on the intestinal epithelium integrity in pig jejunum. In the present study, we hypothesised that bioactive compounds of CHE can affect nutrient digestibility depending on their chemical composition, and that different concentrations of CHE-derived polyphenols differently affect intestine epithelial barrier functionality.

2. Material and methods

2.1. Samples and chemical composition

Three different soy-based meals were used to test the effects of CHEs on soy products diverging in their chemical composition: screw-pressed soybean (SPS), solvent-extracted soybean (SES), and soy protein isolate (SPI). Samples were analysed for dry matter

Table 1
Gross chemical composition of screw-pressed soybean meal (SPS), solvent-extracted soybean meal (SES), soy protein isolate and chestnut extract (CHE). Data are expressed in fresh material in g/kg.

	SPS	SES	SPI	CHE
Dry matter	904.4	877.5	941.4	906.5
Ash	56.6	64.5	45.3	9.3
Crude protein	451.7	441.8	864.3	9.9
Nitrogen	72.3	70.7	138.3	n.a.
Ether extracts	55.1	20.4	30.3	n.a.
Crude fibres	48.7	70.1	1.6	1.5
Hydrolysable tannins	n.a.	n.a	n.a	560

Abbreviations: SPS = screw pressed soybean; SES = solvent extracted soybean; SPI = soy protein isolate; CHEs = chestnut extracts

(DM) and ash content by grinding them to pass through a 1-mm screen, then heating at 105 °C for 3 h, followed by dry-ashing at 550 °C. The mineral content of the dry ashes was measured by an inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 7300 DV; Perkin-Elmer, Schwerzenbach, Switzerland) (European Standard EN 155510:2008). The crude protein (CP) content, calculated as nitrogen (N) x 6.25, where N was determined using the Dumas method (ISO, 2008). The ether extract (EE) was obtained by petroleum ether extraction after acid hydrolysis (ISO, 1999), followed by the removal of the solvent under reduced pressure. The crude fiber (CF) content was determined gravimetrically (ISO, 2000) by incineration of residual ash after acid and alkaline digestions using a fiber analyzer (Fibretherm Gerhard FT-12, C. Gerhardt GmbH & Co. KG, Konigswinter, Germany). The HT content in the CHE was determined as described by Johnson et al. (2014). The chemical composition of the SPS, SES, SPI and CHE samples was obtained by two replicates and is reported in Table 1.

2.2. Digestion of the protein isolate and the soybean meal extracts using the INFOGEST protocol

The CHEs (tannins 560 g/kg) were subjected to the in-vitro gastrointestinal INFOGEST protocol (Minekus et al., 2014). The enzymatic activities and bile concentration were measured before the digestion experiment per the assays described in the harmonized protocol (Minekus et al., 2014). Each substrate of SPS, SES and SPI containing 0.04 g protein was digested without (SPS-, SES-, SPI-) and together with 30 g/kg, as fed CHE (SPS+, SES+, SPI+). This inclusion level was derived from a previous experiment with growing-finishing pigs in which the dietary supplementation of 30 g/kg CHE reduced the intestinal absorption of androstenone undergoing enterohepatic recirculation in entire male pigs (Tretola et al., 2019). In brief, 1 mL of water was added to each sample and mixed with 1 mL of simulated salivary fluid (pH 7) containing amylase (300 U/mL of digesta, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 2 min. Then, 2 mL of simulated gastric juice (pH 3) containing pepsin (2000 U/mL of digesta, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were added and incubated for 120 min. Subsequently, 4 mL of simulated intestinal juice (pH 7) containing pancreatin (100 U trypsin activity/mL of digesta, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and bile (2.5 mmol/L of total digesta, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were added and incubated for 120 min. The digestion was performed at 37 °C under constant gentle mixing on a rotating wheel. Digestion was stopped after 240 min using the protease inhibitor 4-(2-aminoethyl)benzensulfonyl fluoride (AEBSF, trademark Pefabloc®, 500 mmol/L, Roche, Basel, Switzerland). Immediately after stopping digestion, all samples were snap-frozen in liquid N and stored at − 20 °C until further analysis. The digests were then split by centrifugation (850 g x at 4 °C for 15 min) into a soluble and insoluble fraction by collecting the supernatant and the pellet, respectively.

2.3. In-vitro digestibility

The determination of the DM and CP through in-vitro digestibility of SPI-, SPS-, SES-, SPI+ , SPS+ , and SES+ was performed in duplicate. The heat-processed wheat was used as an internal control of the in-vitro digestion. The digestion enzymes alone have also been used (or incubated and used) as blank, as described by Boisen and Fernández (1997). Briefly, 1 g of each sample (SPS, SES and SPI with or without CHEs, the heat-processed wheat and digestion enzymes alone) was mixed with 0.1 M phosphate buffer (pH 6.0). Subsequently, the pH of the solution was lowered with 10 mL of 0.2 M aqueous hydrochloric acid and then fine-adjusted to a final pH of 2.0. Then, 1 mL of freshly prepared pepsin (P7000 Sigma-Aldrich, St. Louis, MO, USA) solution (10 mg mL^{-1}) was added. To minimize bacterial fermentation during digestion, a 0.5 mL chloramphenicol solution (5 mg mL^{-1} ethanol) was added to the mixture. The bottle was placed in a shaking water bath at 39 °C for 6 h (simulation of the gastric phase). Subsequently, 10 mL of 0.2 M phosphate buffer at pH 6.8 was added, followed by 5 mL of 0.6 M NaOH, and the pH was adjusted to 6.8 with 1 M HCl or 1 M NaOH. In addition, 1 mL of freshly prepared pancreatin (P3292; Sigma–Aldrich, Buchs, Switzerland) solution (50 mg/mL) was added to the mixture. The bottle was placed in a shaking water bath at 39 °C for 18 h (simulation of the small intestinal phase). To enable the precipitation of undigested soluble proteins, 5 mL of 20% sulphosalicylic acid was added and the bottle was kept at room temperature for 30 min. The undigested fraction (UF) was then collected in a filtration unit by using dried and pre-weighed glass filter crucibles (d: 3 cm; pore size: 40–90 pm) containing about 0.5 g Celite as a filter aid. All material was then transferred with 1% sulphosalicylic acid to the crucible. After consecutive washings with 2 × 10 mL of ethanol and acetone, respectively, the undigested residues were dried at 80 °C overnight. The in-vitro DM and CP digestibility (IVDMD and IVCPD) was calculated as follows:

IVDMD = (sample DM – sample UF of DM) / (sample DM)·

IVCPD = (sample CP – sample UF of CP) / (sample CP)·

2.4. Quantification of polyphenols

The polyphenol content in samples was quantified using Folin–Ciocalteu's method, previously described by Singleton and Rossi (Singleton and Rossi, 1965), and applied with some modifications. The undigested samples (SPS, SES, SPI, and CHE) were solubilized in HPLC-grade water (VWR International, Fontenay-sosu-Bois, France) to a final concentration of 5 mg/mL, sonicated and filtered with 0.45 μm PP filters (VWR International, Fontenay-sosu-Bois, France). The digested samples and the digestive fluids (used as blank) were centrifugated at 10,000 g for 10 min and filtered with 0.45 μm PP filters. Aliquots of 100 μL of suitably diluted samples, water or digestive fluids (used for blank) were mixed with 0.5 mL of 0.2 N Folin–Ciocalteau's reagent (VWR International, Fontenay-sosu-Bois,

France) and 0.4 mL of 7.5% Na_2CO_3(aq) (Sigma-Aldrich, St. Louis, USA). After 30 min in the dark, the absorbance was measured at 765 nm in a UV–visible spectrophotometer (Varian Cary 50 SCAN, Palo Alto, California, USA) The polyphenol content was quantified using a calibration curve of gallic acid (VWR International, Fontenay-sosu-Bois, France) (range: 5–50 μg/mL) and the results were expressed as equivalents of gallic acid (GAE) in mg/g. The interference of the digestive fluids used for in-vitro digestion in the final quantification of polyphenols has been considered, subtracting the absorbance of the blank (containing only digestive fluids) from that of the other samples.

2.5. Untargeted metabolomics

2.5.1. Sample preparation

One mL of each sample after having been enriched with internal standard (proline D3, final concentration 0.1 ug/mL) was mixed with 1 mL of cold extraction solvent (MeOH:H2O, 80:20). The sample was homogenized by vortex, and left 2 h under − 20 °C, sonicated and centrifugated. 100 μL were resuspended in 900 mL of the initial HPLC mobile phase. The resultant solution was diluted (1:10) in the initial HPLC mobile phase (95% water acidified with 0.1% HCOOH and 5% methanol) and 5uL was injected. All samples were processed in two technical replicates.

2.5.2. HPLC-HRMS profiling and metabolomics approach

All analyses were conducted by using Vanquish HPLC instrument (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Restek RP column. The separation gradient was HCOOH in water and methanol at 0.1%.

HRMS determinations were set in positive and negative mode by using Exploris HRMS (Thermo Scientific, San Jose, CA, USA). Full scan (FS) with resolving power 120.000 (two scan ranges of m/z 70–800 and 800–2500) was used for screening of compound profiles. Fragmentation was obtained with the following parameters: full scan data-dependent acquisition (FS-dd-MS2); resolving power 60.000 and 17.500 for FS and dd-MS2 and collision energy (NCE) set at 20, 30, and 40 eV. Metabolomic workflow was obtained by using Compound Discoverer (CD) 3.3 software (Thermo Fisher, MA, USA) for compound identifications and statistical evaluation as also described in our previous researches (Pavlovic et al., 2020; Leoni et al., 2021). Putative identification of metabolites was obtained by CD workflow chosen as a combination of a few different assets: a mzCloud match score higher than 80% and the same identification being proposed by at least one external web database: Human Metabolome platform HMDB (https://hmdb.ca/), Kyoto Encyclopedia of Genes and Genomes (KEGG), (https:// www.genome.jp/kegg), Pubchem (www.pubchem.com) or Small Molecule Pathway Database (SMPDB) (http://smpdb.ca). MzCLoud (https://www.mzcloud.org and Chempider https://www.chemspider.com), was also used to recognise compounds from procedural blank and medium, thus particularly critical for identification of polyphenolic and flavonoids. If mass fragmentation pattern did not correspond to any of databases software, manual verification of fragmentation pattern program was achieved using ChemDrow software or it was compared with recent publications (Silva et al., 2021). Differential analysis was performed as a part of the CD workflow and the resulting statistical evaluation was expressed by Hierarchical Cluster processing, Principal Component Analysis, and Metabolic Pathway schematization.

2.6. Cell cultures

The IPEC-J2 were maintained in DMEM/Ham's F12 supplemented with 10% Porcine serum (Fisher Scientific, Leicestershire, VS), 50 U/mL penicillin, and 50 U/mL streptomycin. Cells were differentiated on Transwell™ permeable support (Corning, New York, USA) as described previously (Schmidt et al., 2008).

2.7. Cytotoxicity of INFOGEST Digestive Juice and effects of digested CHE on IPEC-J2 viability

Before running the proliferation and epithelial cell integrity assays, an evaluation of the toxicity of the digestive juice on the IPEC-J2 cells was tested. INFOGEST juice (without samples) was diluted in DMEM/Ham's F12 supplemented with 1% porcine serum. Then, the different dilutions obtained were incubated with IPEC-J2 cells for 4 h and 24 h at 37 °C and 5% CO_2. The viability of the cells exposed to digestive INFOGEST juices was optically evaluated and further assessed using the 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyl-2 H-tetrazolium bromide assay (Kumar et al., 2018).

Culture of differentiated IPEC-J2 cells, was employed to determine changes in viability after addition of the SPI-, SPS-, SES-, SPI+ , SPS+ and SES+ samples digested using the previously described INFOGEST protocol. The viability was tested by measuring the integrity of the monolayer, determining the trans-epithelial electrical resistance (TEER) using an EVOM2 epithelial voltmeter (World Precision Instrument, Sarasota, FL, USA).

2.8. Ex-vivo experiments on pig jejuna

The Swiss Cantonal Committee for Animal Care and Use (Fribourg, Switzerland) approved all procedures involving animals (2018_30_FR).

2.9. Experiment 1: response of the jejunum to different digested CHE dilutions

Seven Swiss Large White pigs housed at the Agroscope animal facilities were used. Only females were selected to avoid the sex

effect. The pigs were reared in pens with the slatted metal floor, with ad libitum access to a low-crude-protein grower-finished diet and tap water. The ambient temperature was maintained at around 24 °C. No welfare-related interventions were needed before, during, or after the grower-finisher period. Animals were slaughtered at 171 ± 2.8 d of age (mean and SD) at the research station abattoir after being fasted for approximately 15 h.

Eight jejunal samples per pig were excised starting from the third meter distal to the pylorus. The outer muscular layer was stripped and then tissues were immediately mounted between the two halves of an Ussing chamber (Physiologic Instruments, San Diego, United States). Each segment was mounted in a different Ussing chamber and bathed on its mucosal and serosal surfaces (exposed area 1.0 cm^2) with the corresponding Kreb's Ringier buffers (KRB).

After a stabilization period of 30–40 min, two jejunal samples per pig were randomly assigned to one of the different dilutions (1:4, 1:8, 1:16 in apical KRB) of digested CHE, apically added for 4 h. The remaining two jejunal samples per pig were used as a negative control (KRB without CHE). These dilutions were chosen according to the cytotoxicity assay results obtained from the IPEC-J2 cells. For each CHE dilution and negative control, two replicates were used for each independent experiment. After a 30–40 min equilibration period, the baseline of short-circuit current (Isc, in mV) values was measured. The TEER was also measured at 2 min intervals under current clamped conditions. The TEER was determined at an applied current of 100 mA, and the Isc was calculated using Ohm's law ($R = V/I$). Active uptake was evaluated according to electrical changes in the Isc. The total active transport through the tissue was verified by monitoring the change in Isc (ΔIsc), which was representative of ion flux, and thus, active transport within the jejunal tissue. After a run of 4 h, forskolin (10 μmol/L, Sigma-Aldrich, St. Louis, MO, USA) was added to the apical compartment to test tissue viability. Data obtained from tissues not responding to forskolin were excluded from statistical analysis. Then, at the end of the experiment, tissues were transferred into CellLytic MT (Sigma-Aldrich, St. Louis, MO, USA) complemented with protease inhibitors for subsequent analysis of TJ gene expression.

2.10. Experiment 2: evaluation of the effects of digested CHE on intestinal epithelial integrity in jejunum segments incubated with indomethacin

For this second *ex vivo* experiment, eight jejunal tissues from seven pigs of the same breed, sex, and BW described in Experiment 1 were used. After a stabilization period of 30 min, two jejunal samples per pig were randomly assigned to one of the four apical treatments: i) dilution 1:16 (in apical KRB) of digested CHE for 3 h. ii) dilution 1:16 in combination with the indomethacin (INDO, 250 μmol * L^{-1}) dissolved in apical KRB. iii) negative control group (KRB without CHE and/or INDO). iv) positive control group (KRB with INDO only). The TEER was continuously measured as described in Experiment 1.

2.11. Western blots

The cells and tissues collected in the in-vitro and *ex-vivo* experiments were lysed in CelLytic M and CelLytic MT (Sigma-Aldrich, Buchs, Switzerland), respectively, complemented with protease inhibitors. After centrifugation at 12,000 g x at 4 °C for 10 min, the protein concentration in the supernatant was determined with Coomassie Plus Assay Reagent (Thermo Scientific, Waltham, USA). Absorbance was read on an Asys UVM 340 microplate reader (Biochrom, Cambridge, UK). Of the total protein extracts, 20 μg were denatured at 95 °C for 5 min, separated via 7% or 10% SDS-PAGE gel (polyacrylamide: 30% [w/v], acrylamide: 0.8% [w/v] and bisacrylamide [37.5:1]); 1.5 M Tris HCl, pH 8.8; 0.4% sodium dodecyl sulfate; 10% ammonium persulfate; and 0.01% tetramethylethylenediamine. Proteins were then blotted on a WesternBright (polyvinylidene difluoride) membrane (Witec, Luzern, Switzerland) at 90 V for 90 min using the Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, USA). The membranes were blocked for 60 min at room temperature with 1x phosphate-buffered saline supplemented with 0.1% Tween 20% and 5% bovine serum albumin (BSA). Membranes were further incubated at 4 °C overnight in the same buffer with primary antibodies (ZO1, OCLN, and CLDN1, Abcam, Cambridge, UK, and Vinculin, Sigma-Aldrich, St.Louis, USA) at the appropriate dilutions. Once washed, membranes were incubated with diluted goat–anti-rabbit or goat–anti-mouse HRP-conjugated (Sigma-Aldrich, St.Louis, USA or in phosphate-buffered saline containing 0.1% Tween 20% and 5% milk powder. Signals were revealed using a Quantum Kit (Witec, Luzern, Switzerland) and quantified with Syngene G: BOX and Gene Tools software (Syngene International Ltd., Bangalore, India). The vinculin signal was used to normalize the relative abundance of tight junctions proteins, its signal being constant between animals and experimental conditions considered (data not shown).

2.12. Statistical analysis

The sample size was justified by statistical power analysis using SYSTAT (2018) (Wilkinson, 2010). The main parameter used was arginine (Arg)-induced ΔIsc in jejunal tissues. These values were obtained by a similar study performed on jejuna in Ussing chambers where the absorption of Arg after gallic acid incubation was tested (Tretola et al., 2021). The other parameters included for the power analysis were: statistical model = one-way ANOVA, number of groups = 3, standard deviation = 0.13, significance level = 0.05, power threshold = 80% and effect size = 0.97. Using these parameters, a sample size of five samples per treatment was requested to test or disprove the hypothesis.

The data were analyzed using SPSS (IBM, 2016) and are presented as mean ± SEM. The in-vitro results were obtained by three technical replicates in three independent experiments. For the Ussing-chamber experiments and the related Western Blot analysis, results were obtained from five experimental units for the dilution (1:8) and six for the remaining treatments. For the *ex-vivo* Experiment 2, the results were obtained from five animals per group. The data were tested for normality using the Shapiro–Wilk test

and analyzed using the ANOVA procedure. Specifically, to compare the effect of CHE inclusion on substrates' in-vitro digestibility, one-way ANOVA was performed ($a = 0.05$), followed by the posthoc Tukey test.

The statistical analysis for the Ussing chamber experiments was performed with the nonparametric Mann–Whitney *U* test, as the data were not normally distributed. Data were tested with a mixed linear model in which the pig was considered a random effect and data were Bonferroni corrected. When necessary, a repeated measurement analysis has been performed. Differences between the control and treatment groups were considered significant at $P < 0.05$.

3. Results

3.1. Chemical composition of soybean-based meals and CHE

The processing technique of the SPS, SES and SPI meals strongly affected their chemical composition (Table 1). The CP of the SPI was twice as high compared to the SES and SPS. The level of EE was greater in the screw pressed SPS meal compared to the SES and SPI, while the solvent extract (SES) had a higher amount of crude fiber compared SPS and SPI meals. CHE had low ash and CP content, a similar CF content compared to the SPI but, as expected, a high amount of HT (Table 1).

3.2. In-vitro digestion of soybean derived products with and without CHE

The addition of CHE reduced both the IVDMD and IVCPD independently of the substrate (Table 2). However, both the IVDMD and IVCPD were reduced to a greater extent when CHE was added to SPI (−8.1% and −8.2%, respectively) compared to SES (−7.2% and −5.5%) and SPI (−3.2% and −2.3%) (D × C interaction, $P < 0.05$).

3.3. Polyphenol quantification after INFOGEST in-vitro digestion

After INFOGEST in vitro digestion, the polyphenol content of SPS, SES, and SPI decreased between − 7.0 and − 17.6% (Table 3). However, the highest total polyphenol content in digested samples was observed in the CHE (88.6 ± 12.6 mg/g), even if those values were reduced by − 83% after in-vitro digestion. Similar range of reductions were observed when CHE was added to the soya-based meals.

3.4. Metabolomics profile of digested Soy extracts

The preliminary analysis of the digestive fluid itself has confirmed this matrix as a complex one that influenced the metabolomic profile of investigated samples. Therefore, the metabolomics workflow was divided into two steps: (I) complete CD analysis of samples including the signals that derive from the digestive fluid. (II) limited CD analysis of samples subtracting digestive fluid substances. The first approach revealed a total of 438 signals whose relative ratio is presented by the heatmap (Fig. 1 A) and PCA score (Fig. 1B).

The SPI- and SPI+ resulted as the samples with the highest concentration of metabolites, while CHE presented the lowest overall concentration (Fig. 1 A). The SPS+ differed significantly from SPS- and was closer to CHE (Fig. 1B).

Other groups were more homogeneous regarding CHE addition (Fig. 1 A). After having analyzed all signals, the putative identification of the most abundant signals was performed, followed by the semi-quantitative evaluation of expressed IS equivalents (Supplementary table S1). As it can be noticed the group of the amino acids was the most abundant class: 16 proteogenic amino acids were identified with arginine, leucine, phenylalanine, tryptophan, tyrosine, and valine as the most abundant while 38 di/tripeptides were identified unambiguously (Supplementary table S1). In addition, among the initial 438 signals, a cohort of more than 200 peptides' structures was established, but with the CD-workflow their sequence and full identification couldn't be achieved probably because of the interfering signals of the digestive fluids.

The CD-workflow gave limited information about the polyphenolic and flavonoids identification due to high interfering signals

Table 2
Effects of 30 g/kg, as fed supplemented chestnuts extract (CHE) on in vitro digestibility of the DM (IVDMD) and CP (IVCPD) of soybean meal products and soy protein isolate.

	Treatment[a]									
Item	SPS		SES		SPI		SEM	P-value[b]		
	−	+	−	+	−	+		S	C	S × C
IVDMD	0.736[a]	0.656[b]	0.762[c]	0.691[d]	0.901[e]	0.869 [f]	0.007	< 0.05	< 0.05	< 0.05
IVCPD	0.911[a]	0.829[b]	0.903[c]	0.848[d]	0.913[a]	0.891[e]	0.001	< 0.05	< 0.05	< 0.05

Values are reported as mean values and standard errors of the means.
Different letters in a row indicate significant differences ($P < 0.05$)

[a] SPS-: screw-pressed soybean meal without CHE; SPS+ : screw-pressed soybean meal with 30 g/kg, as fed CHE; SES-: solvent-extracted soybean without CHE; SES+ : solvent-extracted soybean with CHE 30 g/kg, as fed; SPI-: soy protein isolate without CHE; SPI+ : soy protein isolate with 30 g/kg, as fed CHE

[b] S = substrate (SPS, SES, SPI); C = CHE supplementation (+, -)

Table 3
The level of polyphenols expressed as mg ellagic acid equivalents/g in the undigested and after in vitro digestion with the INFOGEST procedure of screw-pressed soybean meal (SPS), solvent-extracted soybean meal (SES) and soy protein isolate (SPI) un-supplemented or supplemented with chestnut extract (CHE; - / +). Experimental data are expressed as means ± standard deviations.

	SPS		SES		SPI		CHEs
	–	+	–	+	–	+	
mg EAG/g[1]							
Undigested	4.0 ± 0.6	18.8 *	3.3 ± 0.3	18.1 *	6.1 ± 0.7	20.8 *	511.1 ± 27.8
Digested	3.3 ± 0.3	6.1 ± 0.6	3.1 ± 0.4	5.8 ± 0.8	5.7 ± 0.5	8.2 ± 0.2	88.6 ± 12.6
Variation (%)[2]	- 17.6	- 67.5	- 7.61	- 68.2	- 7.00	- 60.3	- 82.7
mg EAG/g CHE							
Undigested[1]	–	8	–	14.8	–	14.7	–
Digested[1]	–	2.8	–	2.7	–	2.6	–
Variation (%)[3]	–	-81.1	–	-81.8	–	-82.3	–

Abbreviations: SPS = screw pressed soybean; SES = solvent extracted soybean; SPI = soy protein isolate; CHEs = chestnut extracts.
[1]EAG: equivalents of ellagic acid
[2]% change in the level of EAG compared to the undigested sample
[3]% change in the level of polyphenols espressed as ellagic acid equivalents/g compared to the undigested sample. The level of polyphenols in the undigested SPS+ , SES+ and SPI+ samples were calculated data based on the polyphenol content of the SPS-, SES- and SPI- samples and the polyphenol content of 30 g/kg, as fed CHE supplementation
Except for the calculated values, results are expressed as the average ± SD of 3 technical repetitions

from the digestive fluids. Consequently, a CD-identification strategy that removed the digestive fluids background was applied. In this manner, it was possible to distinguish the most relevant flavonoids structures with a high level of confirmation certainty. This enabled the reconstruction of two metabolic pathways: 1) genistein precursors and metabolite interrelationship (Supplementary Fig. S1, Table 4) and 2) daidzein-formononetin conversion (Supplementary Fig. S2, Table 5).

3.5. Cytotoxicity and functional assessment of INFOGEST digesta on IPEC-J2

No effects of the five dilutions of the INFOGEST digestive juices on IPEC-J2 viability were observed after 4 h (Fig. 2 A). By contrast, after 24 h the 1:2 (v/v) dilution reduced $P = 0.001$) whereas the 1:4 (v/v) dilution increased ($P = 0.01$) the cell viability. The 1:8, 1:16, and 1:32 (v/v) dilutions did not change the viability compared to the control (Fig. 2B). Consequently, the ratio of 1:8 (v/v) digestion juice/medium was chosen as the optimal dilution rate for further assays.

After full differentiation, digested CHE diluted in culture medium at ratios of 1:8, 1:16, and 1:32 (v/v) were added to the apical chamber and further incubated with cell monolayer for 24 h. As a measure of monolayer integrity, the TEER was monitored before ($t = 0$ h) and after incubation ($t = 24$ h). Compared to the control (586.7 ± 76.4 Ω/cm^2), the TEER expressed as a percentage of initial values at $t = 0$ h incubation was not altered by the three dilutions (Fig. 2 C).

3.6. Ex-vivo mucosal electrolyte transport of digested CHE

The Isc was monitored as a measure of net electrolyte transport across the tissue. In all samples, a consistent increase in Isc over the whole experiment was observed (Fig. 3 A). Compared to the control, the three dilution levels of digested CHE induced no changes in the Isc suggesting a poor or absent ion-mediated active transport of digested CHE polyphenols from the mucosal to the basolateral side of the pig jejunal segments.

3.7. Trans-epithelial resistance in jejuna incubated with digested CHEs

After 60 min but not after 120 and 180 min incubation, the 1:4 (v/v) dilution of digested CHE increased ($P = 0.03$) the TEER values of the jejunum compared to the tissues incubated with 1:16 (v/v) dilution, with intermediate TEER values for the 1:8 and control (CTRL) groups (Fig. 3B).

The repeated measurement analysis revealed no differences in the jejunal TEER within groups over the entire experimental time course.

3.8. Tight junction protein expression in jejuna

On the same tissues utilized for monitoring Isc and TEER over time, the expression of TJ proteins CLDN1, ZO1 and OCLN were investigated. None of the tested digested CHE dilutions affected the protein expression of CLDN1 (Fig. 3 C). However, compared to the control and the 1:4 (v/v) dilution, the 1:8 (v/v) dilution increased ($P < 0.05$) the protein expression of both ZO1 and OCLN (+60%), while the 1:16 (v/v) dilution only doubled ($P < 0.05$) the expression of the ZO1 protein.

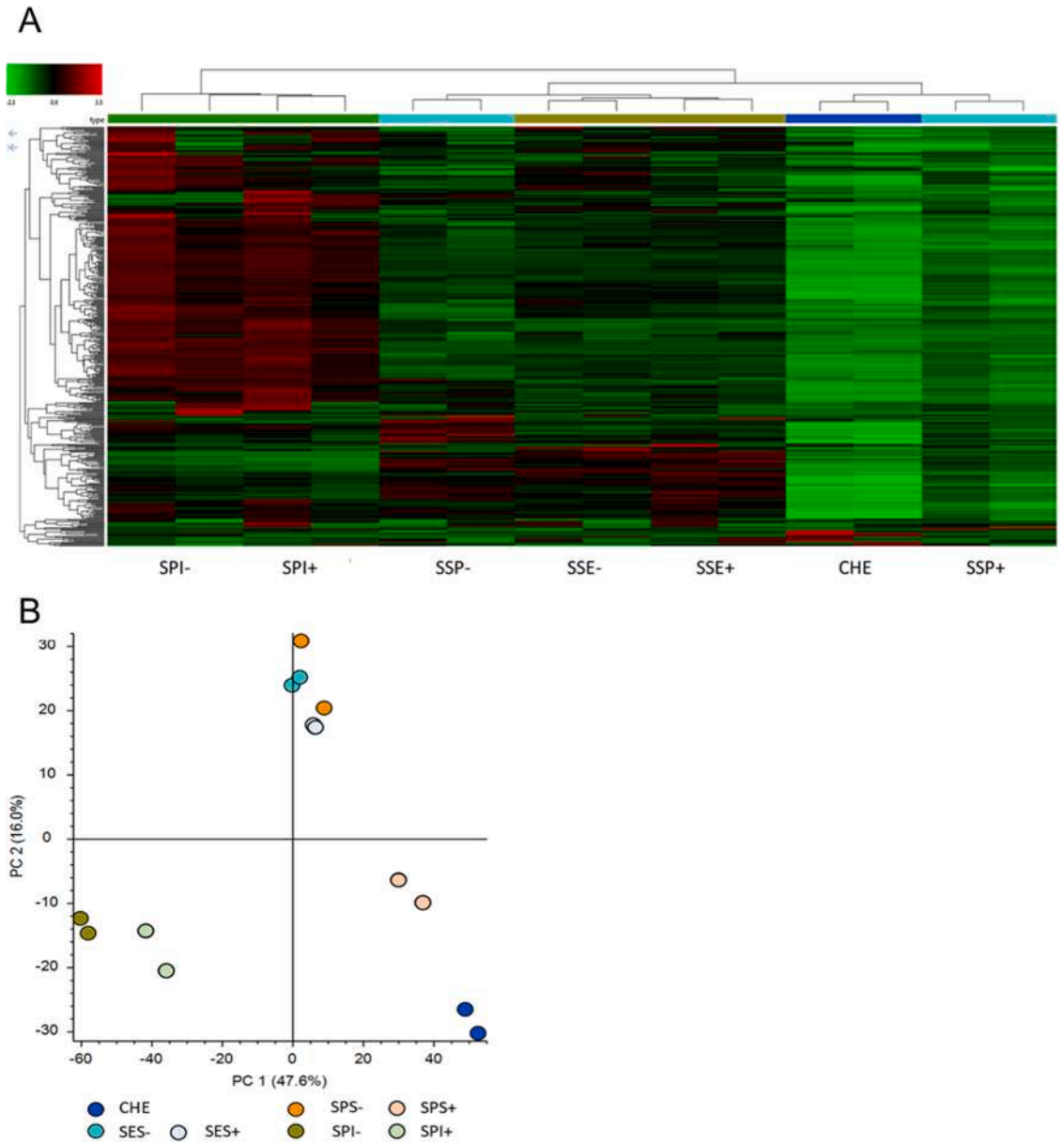

Fig. 1. A) Hierarchical cluster analysis for the 458 signals. Each metabolite is represented by a row and each sample (two replicates per sample) by a corresponding column. The heatmap reflects the differences between the relative amount through normalized chromatographic peak areas between the screw-pressed soybean meal without chestnut extract (CHE) (SPS-), screw-pressed soybean meal with 30 g/kg, as fed CHE (SPS+), solvent-extracted soybean meal without CHE (SES-), solvent-extracted soybean meal with 30 g/kg, as fed CHE (SES+), soy protein isolate without CHE (SPI-) and soy protein isolate with 30 g/kg, as fed CHE (SPI+). The z-color scale indicates normalized peak area value. Red and green colours indicate lower and greater metabolite abundance, respectively. B) Principal Component Analysis (PCA) projection on the distribution of samples of the screw-pressed soybean meal without chestnut extract (CHE) (SPS-), screw-pressed soybean meal with 30 g/kg, as fed CHE (SPS+), solvent-extracted soybean meal without CHE (SES-), solvent-extracted soybean meal with 30 g/kg, as fed CHE (SES+), soy protein isolate without CHE (SPI-) and soy protein isolate with 30 g/kg, as fed CHE (SPI+) concerning PC-1 with PC-2 when signals of 458 detected compounds were used.

3.9. Effect of digested CHE on the indomethacin-induced effect on TEER in jejuna

Given the highest change in ZO1 expression evoked by luminal application of the 1:16 (v/v) dilution of digested CHE, this dilution was chosen to test the hypothesis of a protective effect of digested CHE against the detrimental effects of INDO on jejunum permeability (Fig. 4). The INDO-incubated tissues showed lower ($P = 0.02$) TEER values compared to untreated jejunal samples. However, when tissues were incubated with both INDO and digested CHE (1:16 (v/v) dilution), no differences were observed compared to the control.

Table 4
Genistein precursors and metabolites semi-quantification. Data are presented as Internal standard IS equivalents (ug/mL; mean ± SD for 2 replicates).

	Treatment[1]						
	SPS		SES		SPI		CHE
Item	–	+	–	+	–	+	
Genistein	0.281 ± 0.02	0.243 ± 0.082	0.207 ± 0.058	0.591 ± 0.078	0.05 ± 0.022	0.308 ± 0.042	0.008 ± 0.01
Apigenin	0.034 ± 0.009	0.021 ± 0.002	0.019 ± 0.002	0.069 ± 0.012	–	0.037 ± 0.012	–
Prunetin	0.004 ± 0.001	0.007 ± 0.005	0.006 ± 0.001	0.011 ± 0.003	–	–	0.001 ± 0.002
Biochanin A	0.005 ± 0.002	0.009 ± 0.004	0.007 ± 0.002	0.013 ± 0.005	–	0.004 ± 0.002	0.001 ± 0.001
Naringenin	0.006 ± 0.002	0.002 ± 0.001	0.009 ± 0.002	0.014 ± 0.003	0.003 ± 0.003	–	–
Dalbergioidin	0.004 ± 0.001	0.004 ± 0.001	0.005 ± 0.003	0.009 ± 0.006	0.003 ± 0.001	0.004 ± 0.001	0.003 ± 0.002
2,4′,7-Trihydroxyisoflavone	0.003 ± 0.001	0.004 ± 0.003	0.002 ± 0.001	0.007 ± 0.002	–	0.003 ± 0.001	–
2,5,7-Trihydroxy-4′-methoxyisoflavanone	0.007 ± 0.002	0.018 ± 0.008	0.016 ± 0.022	0.026 ± 0.012	–	–	0.002 ± 0.001

Abbreviations: SPS = screw pressed soybean; SES = solvent extracted soybean; SPI = soy protein isolate; CHEs = chestnut extracts.

Table 5
Daidzein-formononetin and their metabolites semi-quantification. Data are presented as Internal standard IS equivalents (ug/mL; mean ± SD for 2 replicates).

	Treatment[1]						
	SPS		SES		SPI		CHE
Item	–	+	–	+	–	+	
Daidzein	0.585 ± 0.018	0.171 ± 0.052	0.361 ± 0.063	0.433 ± 0.08	0.528 ± 0.002	0.562 ± 0.05	0.121 ± 0.012
Hydroxyhydrodaidzein	–	–	–	–	0.011 ± 0.003	0.006 ± 0.001	–
Hydroxydaidzein	0.006 ± 0.002	0.001 ± 0.005	0.161 ± 0.081	0.011 ± 0.001	0.006 ± 0.001	0.006 ± 0.003	–
Formononetin	0.001 ± 0.001	–	0.002 ± 0.002	0.003 ± 0.001	–	–	–
2-hydroxyformononentin	0.003 ± 0.002	0.001 ± 0.001	0.009 ± 0.002	0.003 ± 0.003	0.002 ± 0.003	0.002 ± 0.002	–

[1] SSP-: screw-pressed soybean meal without CHE; SSP+ : screw-pressed soybean meal with 30 g/kg, as fed CHE; SES-: solvent-extracted soybean without CHE; SES+ : solvent-extracted soybean with 30 g/kg, as fed CHE; SPI-: soy protein isolate without CHE; SPI+ : soy protein isolate with 30 g/kg, as fed CHE
Data (average ± SD) are presented as internal standard (IS) equivalents (ug/mL). Each s; media ± SD for 2 replicates).

4. Discussion

4.1. Effects of CHE on the IVD of soybean-derived products

The main goals of this study were to investigate the effects of CHE on the IVD of three different soybean-based sources, the amount of total polyphenol and metabolites released by those meals, and the effects of CHE-derived polyphenol on epithelial barrier functions in pig jejunum. The SPI- showed the highest IVDMD and IVCPD values. As reported elsewhere (Zhang et al., 2018) the commercial food grade SPI is usually heated at high temperatures of approximately 100 °C and the heating process greatly increases the surface hydrophobicity of the protein (Sorgentini et al., 1995). The same author (Zhang et al., 2018) observed a slight increase in the release of trichloroacetic acid soluble nitrogen during the digestion process in 100 °C-heated SPI compared to untreated SPI, probably because it had the highest DM and CP content, respectively, compared to SPS- and SES-. The lowest IVDMD was observed in SPS. Screw pressing typically consist in a mechanical process, the soybeans are cracked, dried, heated and fed to a mechanical press (screw press), then the resulting flakes are dried and ground. The entire process inactivates the anti-nutritional properties of raw soybeans. While in the solvent extraction process, soybeans are cracked, heated, flaked, and passed through an expander. The expander produces a porous pellet with increased cell rupture and greater density. This makes oil extraction by solvent easier (Dunford, 2012). More severe technical treatment of solvent extraction process might positively affect the nutrient modification and in turn in vitro digestibility of soybean. However, the slight difference (<3%) in in vitro DM digestibility might be interaction between original chemical composition and technical treatment., likely due to screw pressing, given that the DM, ash, and CF content do not explain the obtained result. Although statistical difference have been observed in term of in vitro CP digestibility among three tested soybean based matrix the difference might be considered negligible, since difference were less than 1% out of over 90% in in vitro CP digestibility in all, SPS, SES and SPI.

The addition of CHE, which is a rich source hydrolysable tannins (Girard and Bee, 2020), decreased both the IVDMD and IVCPD of SPS, SES, and SPI. These findings are in line with the common opinion that tannins have anti-nutritional properties in monogastric

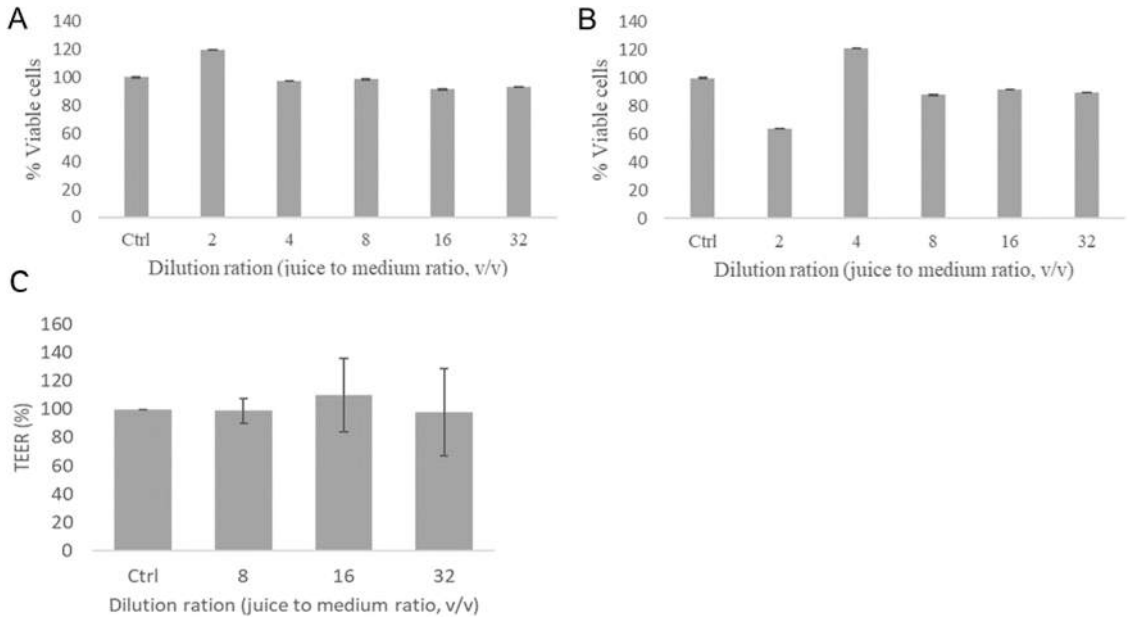

Fig. 2. The effect of digestive juice dilution (control = medium without digestion juices, 2 = 1:2, 4 = 1:4, 8 = 1:8, 16 = 1:16 and 32 = 1:32) on cell viability (expressed as % of the control). The IPEC-J2 cells were incubated for 4 h (A) and 24 h (B) with digestive fluids obtained from INFOGEST in-vitro digestion. Juices did not contain chestnut extracts. Values are means with their standard deviations. C) change in trans-epithelial electrical resistance (TEER; expressed as % change relative to the control) after 24 h incubation of the digested CHE with the IPEC-J2 culture. The TEER values are expressed as a percentage of the initial values at t = 0 h. Dilutions of the digested samples (control = no digested samples, 8 = 1:8, 16 = 1:16 and 32 = 1:32) were chosen based on the results of the cytotoxicity of the INFOGEST digestive juice. Results are based on three technical replicates of three independent experiments. Error bars represent standard deviation.

animals (Redondo et al., 2014). Their ability to bind dietary proteins and digestive enzymes (Redondo et al., 2014) could have led to lower bioavailability of dietary proteins and a block of the enzymes' catalytic sites and their activity, resulting in decreased in-vitro nutrient digestion. However, the impact of the CHE on both the IVDMD and IVCPD differed among the sources, as the extent of the decrease in IVD was greater in SPS compared to SES and SPI. One might hypothesize that these differences were due to different levels of CP, as CHE-derived polyphenols are known to interact with proteins. However, the CP level of SPI was twice as great as the CP level of the other two sources; whereas, the CF level was up to seven times lower in the SPI than the SPS and SES. For this reason, an explanation could be found in the potential inhibitory effects of the CHE on lipase contained in the pancreatic enzymatic components involved in the hydrolysis of the EE (Pinto et al., 2021). Further investigations are needed to clarify the inhibitory effects of CHE inclusion in different meals with different chemical compositions and processing.

4.2. In-vitro digestion and polyphenol quantification

Recent studies have shown that low concentrations of several tannin sources exert antioxidant (Blomhoff et al., 2006) and antibacterial effects (Girard and Bee, 2020). A considerable number of health-promoting effects mediated by plant-derived products could be associated with their polyphenolic content. However, the bioavailability of those compounds is generally unknown or not analysed in most of the studies. Before exerting those effects on the intestine, plants are subjected to the gastrointestinal tract environment, which may highly affect the chemical structure and bioavailability of the plant-derived metabolites. Before the INFOGEST in-vitro digestion, the total polyphenolic content of the soy-derived products (SPS, SES, SPI) ranged from 3.3 and 6.1 mg EAG/g. These values are in agreement with published data, where the total phenolic content of yellow and black soybeans ranged from 2.15 to 6.96 mg EAG/g, respectively (Xu and Chang, 2008). The higher total phenolic content found in the SPI could be related to the low specificity of the Folic–Ciocalteau method. Molecules like sugars, ascorbic acid, and proteins could interact with the reactive agent and the CP content in SPI is twice compared to SPS and SES. However, this method is universally considered an appropriate tool for the rapid evaluation of total phenolic content in different matrices (Di Lorenzo et al., 2017). The total phenolic content in CHE was 511.1 ± 27.8 mg EAG/g, in agreement with recent observations in chestnut shells where values of 590.2 ± 1.7 mg EAG/g have been found (Sorice et al., 2016). In our study, after the INFOGEST in-vitro digestion, the total polyphenol content in CHE dropped by 83% compared to the undigested sample. It is interesting to note that, when CHE was included into the soy-derived products, the decrease in the amount of polyphenols after digestion was markedly lower ranging from − 60.3% to − 68.2% in the SPI and SES, respectively. However, when subtracting the amount of phenols in the SPS-, SES- and SPI- samples, the percentage of reduction in the level of polyphenols derived from CHE ranges from − 81.2 to − 82.7 mg EAG/g. This are similar values observed for the CHE samples. Tannins are a complex structure and further investigations are needed to clarify the nature of the reduction in polyphenol content and to what

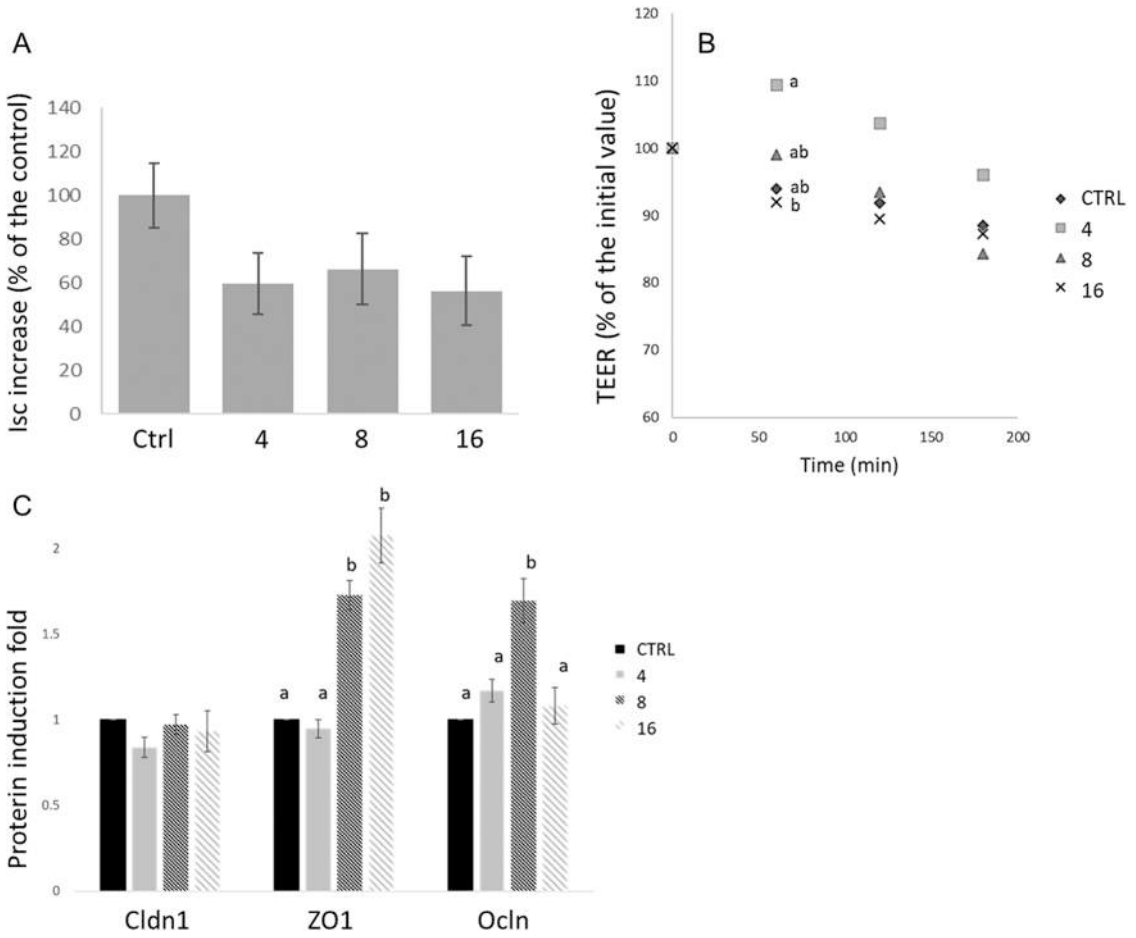

Fig. 3. A) Changes in the short current circuit (Isc, expressed as % change of the control) after 4 h evoked by luminal application of 1:4, 1:8, and 1:16 dilutions of digested chestnut extracts (CHE) (v/v) (sample to apical Kreb's Ringier buffers) to the porcine intestinal mucosa. Values are percentages of the control with their standard errors of five independent experiments. B) Changes in trans-epithelial resistance (TEER; expressed as % of the basal TEER) induced by luminal application of three dilutions (4 = 1:4; 8 = 1:8; 16 = 1:16) of digested chestnut extracts (CHEs, v/v) (sample to apical Kreb's Ringier buffer) to the porcine intestinal mucosa. Different letters indicate a significant ($P < 0.05$) statistical difference. Data for each dilution were obtained in five (n = 5) independent experiments. C) Tight junction protein induction fold in tissue incubated for 180 min with three digested chestnut extracts (CHEs) dilutions (4 = 1:4; 8 = 1:8; 16 = 1:16 v/v sample to apical Kreb's Ringier buffer). CLDN1: claudine-1; ZO1: zonula occludens-1; OCLN: occludin. Data are ls-means ± standard deviations. Data were obtained by at least five independent experiments.

extent this structure is changed after digestion. This would help to understand why the bioavailability of CHE compared to soybean products' polyphenols is low and why the IVD of SPI, SPS and SES differed. Different studies revealed that digestion, especially during the intestinal phase, could affect both the stability and bioavailability of polyphenols. As shown by Gunathilake et al. (2018) significant pH changes, body temperature and gut microbiota composition can alter the chemical structure of the phenolic compounds, together with the activity of the digestive enzymes that can accelerate the HT hydrolysis during digestion. The spectrophotometric method used for the quantification of phenolic compounds is a useful tool for the rapid evaluation of total phenolic content; however, it cannot provide information about the chemical structures of the metabolites present in samples after in-vitro digestion. Thus, the detection limit of the technique used in this study for polyphenol quantification after digestion must be considered, given its limitation.

4.3. Metabolic profile of in vitro digested soy-based substrates with or without chestnut

Independently of the CHE presence, the substrate strongly influenced the presence of metabolites quantified after the INFOGEST in vitro digestion. The SPI was the substrate with the highest metabolites concentration. In general, the group of amino acids was the most abundant class which agrees with the literature (Silva et al., 2021). It is interesting to note that the CHE addition slightly influenced the metabolic profile of digested SPI and SES, but significantly affected the outcome of the digested SPS by reducing the number of released metabolites. Accordingly, the metabolic profiles of CHE and SPS+ were almost comparable. As can be noticed in Supplementary table S1, there was a sort of cumulative effect in SPI and SES samples when CHE was added, regarding at least the amino acids profile. In contrast, in the SES+ samples the abundance of all the amino acids decreased. Ginestein and daidzein were the most abundant

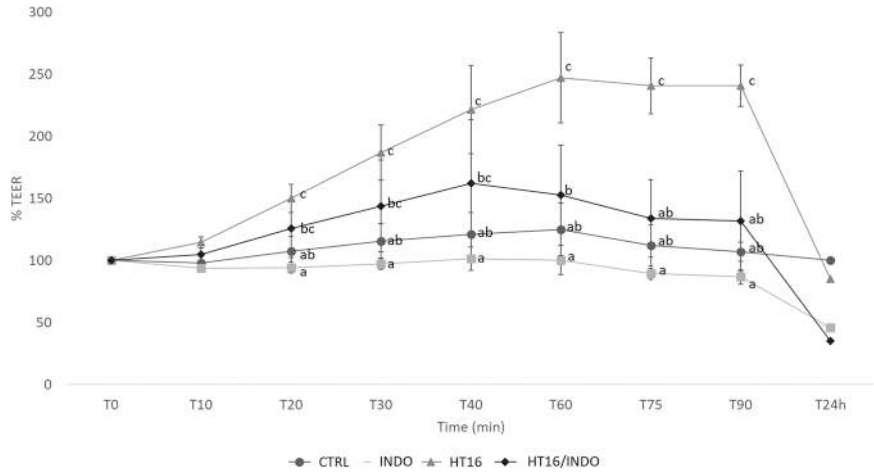

Fig. 4. Effect of indomethacin (INDO, 250 μM) on the barrier function of pig jejunum and protection by digested CHE at a 1:16 dilution (v/v) (sample to apical Kreb's Ringier buffer). 16/INDO = digested CHE diluted 1:16 (v/v) (sample to apical Kreb's Ringier buffer) in the presence of indomethacin; 16 = digested CHE diluted 1:16 (v/v).

isoflavones, in accordance with previous reports in the literature on soybeans and soy products (Ho et al., 2002; Lee et al., 2015). To note that CHE increased the amount of genistein in SPI+ and SES+ but not in SPS+ samples. By contrast daidzein level remained unchanged between SP- and SPI+ and SES- and SES+ whereas the level decreased SPS+ compared to SPS- samples. Summarizing, CHE decreased the nutritional properties of SPS and the bio-accessibility of specific isoflavones after digestion.

4.4. Chestnut-derived metabolites and intestinal epithelial integrity

In this study, we used CHE-derived metabolites obtained after gastric and small intestinal in-vitro digestion to determine their effects on epithelial integrity and their potential protective effects. An intact intestinal barrier is mainly related to a correct expression of TJ proteins, which can separate the intestinal lumen by the systemic compartment of the organism, blocking the paracellular penetration of potentially dangerous microorganisms or toxins (Wijtten et al., 2011). The alteration of this epithelial integrity represents a well-known risk factor for several intestinal diseases that could lead to reduced overall health (Wijtten et al., 2011). Among the proteins involved in the formation of the tight junctions, the CLDN1, OCLN, and ZO1 proteins are probably the most investigated. In accordance with the literature (Tretola et al., 2021), an acute exposition to a mixture of polyphenols obtained by in-vitro-digested CHE affected both the TEER and the expression of the selected proteins involved in TJ formation. Specifically, the 1:4 (v/v) dilution increased the TEER values after 60 min of treatment, compared to the control group, while the 1:8 (v/v) and 1:16 (v/v) dilutions increased the protein expression of both ZO1 and/or OCLN but not the TEER. It is not clear why increased protein expression of ZO1 and OCLN observed in the small intestine incubated with the 1:8 and 1:16 dilution does not correspond to an increased TEER in the Ussing-chamber experiment. An explanation could be related to the regulation of TJs via ion channels. It has been demonstrated that the sodium-hydrogen exchanger (NHE) family can regulate paracellular barrier functions and TJ function *ex vivo* by binding the actin cytoskeleton (Slifer and Blikslager, 2020). Similarly, we recently found that only low concentrations of punicalagin and gallic acid can modulate the expression of different intestinal transporters both in vitro (Biolley et al., 2019) and *ex vivo* (Tretola et al., 2021). These data suggest that the lowest concentrations of CHE-derived polyphenols indirectly increased TJ protein expression without affecting the TEER of jejuna segments because of their effects on other mechanisms that increased the epithelial membrane permeability, such as an increased expression of members of the NHE protein family. The higher ZO1 and OCLN protein expression observed in this study could also explain the protective effects of the polyphenol mixture in the jejunal segments exposed to INDO. This conclusion can be drawn from the fact that intragastric INDO administration in vivo increased the intestinal paracellular permeability, which is mainly determined by TJ integrity (Gotteland et al., 2001). In our study, INDO decreased TEER in the jejunum, but this effect was counteracted by the presence of CHE-derived polyphenols, consistent with the higher ZO1 protein expression observed. These results suggest a protective role of digested HT against the action exerted by INDO in pig jejunum samples. Similar results have already been observed in Caco-2 cells exposed to INDO, where polyphenols protected the epithelial barrier function through the increased expression of OCLN and ZO1 proteins (Carrasco-Pozo et al., 2013). It is important to note that this protective effect has been tested with the lowest concentration of polyphenols (1:16 dilution), since concentrations of polyphenols in the swine or human colon tend to be low due to modification by commensal microbiota. In addition, the highest change in TJ protein expression, specifically that of ZO1, was evoked by luminal application of the lowest concentration of digested CHE. These results suggest that CHE, once digested, could exert protective effects against functional damage induced by external factors, such as INDO.

5. Conclusions

Chestnut extracts exert some anti-nutritional effects on the feed digestibility when included at 30 g/kg, as fed in soy-based meals. Their effects on the in-vitro digestibility vary according to the chemical composition of the meal. The chestnut extracts are an important source of polyphenols, and digestive processes affect their bioavailability. The chestnut extracts also influence the amino acids profile of the soy-based product depending on the processing conditions. The bio-accessible polyphenols after chestnut extracts in-vitro digestion exert protective effects on the intestinal epithelial cell integrity ex vivo when used at low concentration. Further studies are necessary to clarify the involvement of the chestnut extracts -derived polyphenols on paracellular permeability when not affected by tight junction proteins.

Ethics approval

The Swiss Cantonal Committee for Animal Care and Use (Fribourg, Switzerland) approved all procedures involving animals (2018_30_FR).

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CRediT authorship contribution statement

Marco Tretola: Conceptualisation, investigation, methodology, data curation, writing—original-draft preparation. **Paolo Silacci:** Conceptualisation, visualization, Writing- Reviewing and Editing, supervision and validation. **Raquel Sousa:** methodology, data curation. **Francesca Colombo:** methodology, data curation. **Sara Panseri:** methodology, data curation. **Matteo Ottoboni:** methodology, data curation. **Luciano Pinotti:** Reviewing, Editing, visualization. **Giuseppe Bee:** Conceptualisation, Writing- Reviewing and Editing, supervision and validation. All the authors have read and agreed to the published version of the manuscript.

Data Availability

Raw data is available upon request.

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Declaration of interest

None.

Authors statement

All recommendations from the editor and the reviewers were taken into account. In the revised version, the changes are marked in yellow to make the review process easier for the editor and the reviewers.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2022.115501.

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