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Abbreviations: AAI, AntiAtherogenic Index; AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; ABTS, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt; ALT, Alanine Transaminase; AMPK, Adenosine Monophosphate-Activated Protein Kinase; AST, Aspartate Transaminase; BR, Bilirubin; cDNA, complementary DeoxyriboNucleic Acid; CTR, Control; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, Dry Weight; ELISA: Enzyme-Linked Immunosorbent Assay; FRAP, ferric reducing antioxidant power; GSH, reduced Glutathione; HDL-C, High-Density Lipoprotein Cholesterol; H&E, Hematoxylin-Eosin; HFD, High Fat Diet; HO-1, Heme Oxygenase-1; HPLC-DAD, High Performance Liquid Chromatography with Diode-Array Detection; IL-6, Interleukin-6; LDL, Low-Density Lipoprotein; LDL-C, Low-Density Lipoprotein-Cholesterol; LOD, Limit Of Detection; MDA, Malondialdehyde; NAFLD, Non-Alcoholic Fatty Liver Disease; NASH, Non-Alcoholic SteatoHepatitis; NRF-2, Nuclear factor erythroid 2-related factor 2; NF-κB, nuclear factor-κB; ORAC, Oxygen Radical Absorbance Capacity; PCA, Principal Component Analysis; PPAR-α, Peroxisome Proliferator-Activated Receptor-α; PVDF, PolyVinylidene DiFluoride; QE, Quercetin Equivalent; RNA, RiboNucleic Acid; ROS, Reactive Oxygen Species; RT-qPCR, Real-Time quantitative Polymerase Chain Reaction; SCFAs, Short Chain Fatty Acids; SD, Standard Deviation; SREBP-1, Sterol Regulatory Element-Binding Protein-1; STZ, Streptozotocin; TC, Total Cholesterol; TNF-α, Tumor Necrosis Factor-α; TG, Triglycerides; Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; VLDL-C, Very Low-Density Lipoprotein-Cholesterol

Abstract

Herein we characterized the bioactive metabolites of the aqueous extract of Kavolì®, a commercial product composed of a mixture of *Brassica oleracea* leaves, and assessed its potential ameliorating effects in a rat model of non-alcoholic fatty liver disease (NAFLD). Kavolì® extract showed high levels of bioactive compounds and strong *in vitro* antioxidant activities. Chlorogenic and neochlorogenic acids were identified as the most representative polyphenols. The administration of brassica extract to steatotic rats significantly ameliorated the levels of blood lipids and transaminases, and lipid content and inflammatory markers in liver. Oxidative stress parameters were significantly improved in both liver and brain of steatotic rats. Moreover, plasma and feces levels of short chain fatty acids (SCFAs) were bring back close to control values by Kavolì® treatment, in spite of high fat diet/streptozotocin (HFD/STZ)-induced alterations. The efficacy of Kavolì® in treating hypercholesterolemia, reducing the level of inflammation and cardiovascular disease biomarkers, steatosis and oxidative stress parameters, as well as the ability in modulating SCFAs levels is probably related to the bioactive compounds of the water extract administered to the rat model of NAFLD. In particular, the ameliorating effects are largely attributable to the high content in polyphenols observed in our study.

Keywords: *Brassica oleracea*, NAFLD, inflammation, oxidative stress, high fat diet, Wistar rats

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) arises as infiltration of triglycerides into hepatocytes, and includes a wide spectrum of hepatic pathological conditions, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). The latter is characterized by a series of inflammatory processes leading to hepatic fibrosis, cirrhosis as far as hepatocellular carcinoma (Marra et al., 2008; Juárez-Hernández et al., 2016). In addition, NAFLD is closely associated with other metabolic disorders such as type 2 diabetes mellitus, since some studies suggest that up to two thirds of diabetic patients have NAFLD (Chalasani et al., 2018). The constant increase in NAFLD prevalence has made it the most common chronic liver disease worldwide affecting on average more than 30% of the adult population, thus representing a global public health issue (Mitra et al., 2020). Although many aspects are still to be clarified, it is widely accepted that oxidative stress plays a key role in the development of NAFLD (Lee et al., 2019). In fact, an excessive production of reactive oxygen species (ROS)

occurs in this pathologic condition, caused by both decreased activity of ROS scavenging mechanisms and increased mitochondrial ROS production, resulting in the formation of lipid peroxides, such as malondialdehyde (MDA), as by-products of oxidative stress induction. The over-accumulation of ROS and peroxides can determine hepatocytes damage due to phospholipid membrane disruption, protein carbonylation and mitochondrial dysfunction, together with nuclear factor-κB (NF-κB)-mediated secretion of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. Consequently, Kupffer cells and following stellate cells can be activated leading to the production of collagen, thereby promoting fibrosis and NASH (Lee et al., 2019; Li et al., 2021). Hence, the suppression of liver oxidative stress and inflammation is a critical approach for treating NAFLD. However, no standard guidelines for NAFLD pharmacological therapy have been approved to date, and lifestyle intervention involving diet and body weight improvements still remains the mainstay for NAFLD prevention and treatment (Bril and Cusi, 2017). Therefore, nutraceuticals, intended as dietary food supplements useful to prevent diseases, improve health or delay aging, are gaining increasing attention due to their therapeutic and nutritional potential (Sachdeva et al., 2020). Consequently, food manufacturers are developing products of natural origin capable of exhibiting such properties, so as that may result attractive to consumers. Among these food products, *Brassicaceae* can play a role of primary importance. *Brassica oleracea* includes many crop varieties of primary economic importance, such as cauliflower, kale, Brussels sprouts and broccoli, covering an acreage close to 30% of the entire vegetables production in the USA (Mageney et al., 2017). Several studies have demonstrated that dietary intake of *Brassicaceae* can play a relevant role in the prevention of a broad spectrum of diseases. In fact, the antioxidant, anti-inflammatory and lipid-lowering effects of different variety of *Brassicaceae* were previously proved in rats fed a high fat diet (HFD) (Lee et al., 2009; Melega et al., 2013; Dal et al., 2018). Furthermore, earlier studies linked *Brassica* vegetables intake to reduced risk of chronic diseases such as cardiovascular disease and cancer (Chu et al., 2002; Uhl et al., 2004). Even though it is still unclear whether beneficial effects are due to single classes of molecules or to the synergistic interactions of several compounds within the whole vegetable, the phytochemical composition is directly related to these widely recognized preventive powers. In particular, the high content in bioactive compounds like polyphenols and glucosinolates is considered the principal responsible for functional properties of *Brassicaceae*, mainly consisting in neuroprotective power, anti-carcinogenic, antioxidant, hypoglycemic, lipid-lowering and anti-inflammatory capacities (González-Castejón and Rodriguez-Casado, 2011; Ramirez et al., 2020). The most representative phenolic compounds present in *Brassicaceae* family are flavonoids, and among these the most important group corresponds to the flavonols, with qualitative and quantitative profiles varying significantly among different species (Ramirez et al.,

2020). Nuclear factor erythroid 2-related factor 2 (Nrf2) has been identified as a new therapeutic target for NAFLD alleviation by Brassica species, since growing evidence indicates that dietary phenolic compounds are able to activate this transcription factor, protecting hepatocytes from oxidative stress, inflammation and lipotoxicity (Li et al., 2021). In the present study, Kavolì®, a commercial product by Consorzio Freschissimi (Venezia, Italy), composed by a mixture of *Brassica oleracea* var. *acephala* leaves, was analysed for chemical components, with emphasis in phenolic compounds. The aqueous extract of Kavolì® was then administered (1 g/kg body weight (BW)) to a HFD-streptozotocin (STZ) induced NAFLD model of rat, previously characterized in our laboratories (Vornoli et al., 2014; Pozzo et al., 2016), to *in vivo* assess its potential antioxidant, anti-inflammatory and hypolipidemic effects and therefore its ability in ameliorating NAFLD pathology. Finally, short chain fatty acids (SCFAs) in plasma and feces were evaluated to test the impact of HFD treatment and verify whether our tested substance is capable to induce a direct modulation of gut microbiota metabolites.

2. Materials and methods

2.1 Chemicals and reagents

All solvents and chemicals were of analytical grade. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), trichloroacetic acid (TCA), perchloric acid (PCA), thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TEP), guanidine HCl, *o*–phthaldehyde, glutathione (GSH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS), ferrous sulfate ($FeSO_4 \times 7H_2O$), iron (III) chloride hexahydrate ($FeCl3 \times 6H_2O$), 2,4,6-tripyridyl-s-triazine (TPTZ), phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4), hydrochloric acid (HCl), streptozotocin (STZ), sodium carbonate, sodium hydroxide, potassium persulfate, Folin-Ciocalteu reagent, catechin hydrate, gallic acid, ethanol, acetate buffer ($CH3COOH/CH3COO-$), phosphate buffer (Na_2HPO_4/NaH_2PO_4), fluorescein sodium salt, ethyl acetate, and methanol from Sigma-Aldrich (St. Louis, MO). All chromatographic analytical standards, including protocatechuic acid, syringic acid, rutin, elaigic acid, cynaroside, daidzein, neochlorogenic acid, chlorogenic acid, vitexin, trans *p*-coumaric acid, trans sinapic acid, trans ferulic acid, rosmarinic acid, resveratrol, apigenin, myricetin, quercetin and kaempferol, were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffer saline (PBS) were purchased from VWR (Radnor, PA).

The fine powdered freeze-dried Kavolì® (Patent number: PD000213236) was supplied by Consorzio Freschissimi (Venezia, Italy), and stored in a dry and dark place at 4°C.

2.2 Test substance preparation

Fine powdered freeze-dried Kavolì® (Consorzio Freschissimi, Italy) underwent serial extractions in bidistilled water (100 mg/ml), through sonication (10 on/off, 3 cycles) at room temperature (25°C) for 2 hours in the dark. The mixture was then centrifuged twice at 3500 rpm for 10 min, and supernatants were lyophilized under reduced pressure ($T < 40^{\circ}C$) and stored at -20°C. For animal treatment in the *in vivo* experiment, lyophilized extract was re-suspended in water to a concentration of 160 mg/ml, in order to administer 1 g/kg BW to rats.

2.3 Determination of bioactive compounds and in vitro antioxidant activities

For component analyses, lyophilized extract was re-suspended in bi-distilled water to a concentration of 50 mg/ml. Total polyphenols were determined by the Folin-Ciocalteu colorimetric method (Gabriele et al., 2015) and expressed as mg of gallic acid equivalents (GAE)/g dry weight (DW). Total flavonoids were quantified using the aluminum chloride colorimetric method and expressed as mg catechin equivalent (CE)/g DW (Gabriele et al., 2015). Total flavonols were measured as previously described by Souid et al. (2016) and expressed as mg quercetin equivalent (QE)/g DW. All assays were performed in triplicate and results are expressed as mean ± standard deviation (SD). The antioxidant capacity of Kavolì® extract was explored *in vitro* using a combination of fluorimetric and spectrophotometric methods, namely the oxygen radical absorbance capacity (ORAC) assay, the ferric reducing antioxidant power (FRAP) assay, and the DPPH and ABTS radical scavenging assays. The antioxidant capacity was quantified using the ORAC assay according to Gabriele et al. (2015). AAPH was used as peroxyl radical generator and fluorescein as a probe. Trolox was used as antioxidant standard. Results were expressed as ORAC units (µmol Trolox equivalents/g DW). The total antioxidant power of Kavolì® extract was determined using the FRAP assay, a colorimetric method based on the reduction of a ferric tripyridyltriazine complex to its ferrous form, as described by Colosimo et al. (2020). Data were translated into the FRAP value (micromolar) using a water solution of Fe(II) in the range of 100-2000 µM $FeSO_4 \times 7H_2O$ for calibration. The DPPH radical scavenging activity was evaluated according to the method described by Boudjou et al. (2013), with some modifications; this anti-radical capacity was expressed as IC_{50} (mg mL^{-1}), corresponding to the extract concentration needed for causing a 50% DPPH inhibition (Gabriele et al., 2017). Finally, the ABTS radical scavenging activity was determined according to the method described by López-Martinez et al. (2009); the antiradical activity was expressed as IC_{50} (mg mL^{-1}), that is the extract concentration required to cause a 50% $ABTS^{\bullet+}$ inhibition. All assays were performed in triplicate and results are expressed as mean ± SD.

2.4 Polyphenols quantification in Kavolì® by High Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD)

Fine powdered freeze-dried Kavolì® was extracted with 20 mL of 80% ethanol (v/v) at room temperature for 2 hours by horizontal shaker Unimax 2010 (Heidolph Instruments, GmbH, Germany). The extract was filtered through Munktell No 390 paper (Munktell & Filtrak GmbH, Bärenstein, Germany) and stored in closed 20 mL PE vial tubes. Prior to HPLC analysis, the extract was filtered trought syringe filter Q-Max (0.22 µm, 25 mm, PVDF) (Frisenette ApS, Knebel, Denmark).

All compounds were determined using an Agilent 1260 Infinity HPLC (Agilent Technologies GmbH, Waldbronn, Germany) with quarternary solvent manager coupled with degasser (G1311B), sampler manager (G1329B), column manager (G1316A) and DAD detector (G1315C). All HPLC analyses were performed on a Purosphere reverse phase C18 column (250 mm × 4 mm × 5 µm) (Merck KGaA, Darmstadt, Germany). The mobile phase consisted of gradient acetonitrile (A) and 0.1% phosphoric acid in bi-distilled water (B). The gradient elution was as follows: 0-1 min isocratic elution (20%A and 80% B), 1-5 min. linear gradient elution (25% A and 75% B), 5-15 min. (30% A and 70% B) and 20-25 min. (40% A and 60% B). The initial flow rate was 1 mL/min. and the injection volume was 10 mL. Column thermostat was set up to 30 °C and the samples were kept at 4 °C in the sampler manager. The detection wavelengths were set up at 265 nm (protocatechuic acid, syringic acid, rutin, elaigic acid, cynaroside and daidzein), 320 nm (neochlorogenic acid, chlorogenic acid, vitexin, trans *p*-coumaric acid, trans sinapic acid, trans ferulic acid, rosmarinic acid, resveratrol and apigenin) and 372 nm (myricetin,quercetin and kaempferol). Data were collected and processed using Agilent OpenLabChemStation software for LC 3D systems.

2.5 In vivo experiment

2.5.1 Animal procedure

The *in vivo* experiment was performed using male (200-230 g BW) Wistar rats as reported in our previous experiments (Vornoli et al., 2014; Pozzo et al., 2016), with some modifications. Briefly, the animals were divided into two groups and maintained on a 12h light/dark cycle in cages at room temperature with the 55% of relative humidity. Both groups received food and drinking water *ad libitum*. In particular, the control (CTR) group (n=5) received standard feed administered in pellet, containing 19.0% of proteins, 6.0% of fibers, 7.0% of minerals and vitamins moisture, 64.0% of carbohydrates and 4.0% of fats (the latter percentage corresponding to the 11% of the diet-deriving energy). The second group received a high fat/cholesterol feed administered in pellet, containing 13.8% of proteins, 4.4% of fibers, 5.1% of minerals and vitamins moisture, 48.7% of carbohydrates and 28.0% of fats, including 2.0% cholesterol, (this 28.0% of fats corresponds to the 55% of diet-

deriving energy), and after 4 weeks the rats were treated with a single i.p. injection of STZ (40 mg/kg BW) (Sigma, St Louis, MO). Since the majority of NAFLD patients have type 2 diabetes and considerable hyperglycemia, the administration of STZ to our experimental rats was aimed to mildly inhibit beta cell function thereby promoting hyperglycemia, in order to model the development and metabolic characteristics of human steatosis in the presence of type 2 diabetes. Sixteen rats, which resulted hyperglycemic with a glucose concentration > 250 mg/dL, underwent HFD diet for further 5 weeks and were randomly divided into two groups: HFD group (n=8) and HFD+Kavolì® group (n=8) (Figure 1). One g/kg BW/day of water extract of Kavolì® was intragastrically administered to rats from HFD+Kavolì® group, while the same volume of water was intragastrically administered to rats from CTR and HFD groups. For calculating the weight increase, animals were weighted both at the beginning and at the end of the experiment. Before the sacrifice, blood samples were taken from each animal of the three experimental groups by cardiac puncture under general anesthesia, and then centrifuged at 4,000 rpm for 15 min to obtain plasma samples for laboratory analysis. The final sacrifice of experimental animals was performed by heart removal after blood collection. Liver tissues were weighted and stored at -80 °C for extraction and quantification of hepatic lipids, evaluation of oxidative stress markers, and total RNA extraction, or stored in formalin at 4°C for histopathological analysis. All animal procedures of the present experiment (Prot. N. 2612) were performed with the approval of the Local Ethical Committee according to the Italian law regulating the use and humane treatment of animals for scientific purposes (decreto legislativo 26/2014), and the European Union Directive 2010/63/EU for animal experiments.

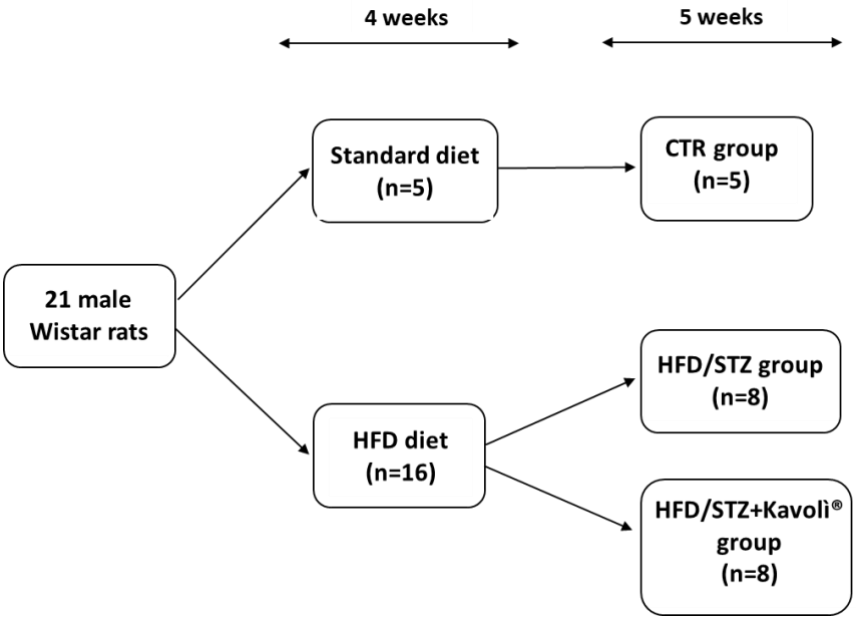


Figure 1. Study design of the *in vivo* experiment

2.5.2 Analysis of biochemical parameters

Plasma analysis were performed using a semi-automatic analyzer for clinical chemistry (model ARCO Biotecnica Instruments, SPA., Italy) for aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin (BR), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C). Low-density lipoprotein-cholesterol (LDL-C) level was derived from the following equation: LDL-C = TC − [(TG/5) + HDL-C] (Friedewald et al., 1972), while plasma level of very low-density lipoprotein-cholesterol (VLDL-C) was derived from the formula: VLDL-C = TG/5 (Bell, 1995). Cardiovascular risk indices were determined using the following formulas: cardio-vascular risk index 1 = TC/HDL-C, and cardiovascular risk index 2 = LDL-C/HDL-C (Ross, 1992). The antiatherogenic index (AAI) was calculated using the formula: AAI = HDL-C × 100/TC − HDL-C (Guido and Joseph, 1992). Glucose level was measured with a glucose meter (Accu-Chek® Roche, Mannheim, Germany) and insulin using a Rat Insulin ELISA kit (Mercodia, Sweden).

2.5.3 Hepatic lipids quantitation

Lipids were extracted and quantified from hepatic tissue following the method described by Folch et al. (1957), with slight modifications. Rat liver tissue was homogenized with equal volumes of methanol and water. The resulting homogenate was subjected to three subsequent extractions in chloroform, followed by two washes with 1 M KCl and water. After complete evaporation and prolonged drying of the chloroform, fat content was weighed and expressed as mg/g tissue.

2.5.4 Oxidative stress evaluation

The concentration of MDA in samples of liver and brain was determined according to Seljeskog et al. (2006), with slight modifications as described in our previous paper (Pozzo et al., 2019); MDA concentration was calculated as nmol/g tissue. The protein oxidation level was determined by the carbonyl protein assay according to Terevinto et al. (2010), with slight modifications (Pozzo et al., 2019); carbonylated proteins concentrations were calculated as nmol/g tissue. GSH levels were evaluated using the method by Browne and Armstrong (1998), slightly modified as reported in Pozzo et al. (2019), and the GSH concentrations were expressed as μmol/g tissue. Heme oxygenase-1 (HO-1) activity was determined by the method of Naughton et al. (2002). Samples were measured by the difference in absorbance between 464 and 530 nm (ε = 40 mM^{-1} cm^{-1}) and HO-1 activity was calculated in pmol/mg prot × min.

2.5.5 Real-time PCR

The isolation of total RNA from frozen rat liver samples (20-30 mg) was performed using the E.Z.N.A.® Total RNA Kit I (OMEGA bio-tek, Norcross, GA, USA), then reverse-transcription by using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, MA, USA). Quantitative Real-Time PCR was executed using the SsoFast™EvaGreen® Supermix (Bio-Rad, CA) in a CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA). Interleukin-6 (IL-6), tumor necrosis factors-alpha (TNF-α), heme oxygenase-1 (HO-1), and β-actin gene primers were designed using Beacon Designer Software (Premier Biosoft International, USA), synthesized by Sigma (St. Louis, MO, USA) and depicted in the Table S1. All genes were assayed in triplicate and the gene expression was calculated by the $2^{-\Delta\Delta CT}$ relative quantification method. The data are expressed as a fold-change of expression levels compared to the control samples.

2.5.6 Histopathological analysis

Liver tissues (n=5) were collected and preserved in a 10% neutral buffered formalin solution. The trimmed specimens were processed and embedded in paraffin blocks according to standard operating procedures of the laboratory. Then, 5 μm sections were cut and routinely stained with Hematoxylin-Eosin (H&E). All the slides were examined under a light microscope (Mark et al., 2007).

2.5.7 Short Chain Fatty Acids (SCFAs) evaluation in plasma and stool content

The blood plasma was separated in a microcentrifuge at 12,000 × g at 4°C for 20 minutes and stored at -70°C until analysis. Plasma samples were extracted using 5 mL of diethyl ether, according to De Baere et al. (2013). After centrifugation (5 min, 3,500 rpm), the supernatant was transferred to another extraction tube and 0.5 mL of 1 M NaOH was added. The samples were extracted again, followed by a centrifugation step. The aqueous phase was transferred to an autosampler vial and 0.1 mL of concentrated HCl was added. After vortex mixing, 10 microliters aliquot was injected onto the HPLC-UV apparatus (Kontron Instruments, Milano, Italy). Chromatographic separation of short chain fatty acids (SCFAs) was tested on Hypersil Gold aQ column (150 mm × 4.6 mm i.d.) with particle sizes of 5 μm (Sercolab, Merksem, Belgium). The HPLC columns were protected by a guard column of the same type. The mobile phase consisted of 20 mM NaH_2PO_4 in HPLC water (pH adjusted to 2.2 using phosphoric acid) (A) and acetonitrile (B). The UV detector was set at a wavelength of 210 nm. The sample concentration was calculated referring to a commercial standard mixture of volatile fatty acids (CRM46975, Supelco, USA) and data processing was performed using Gemini system software (version 1.91).

Fecal sample of each rat was used to determine the concentration of SCFAs, according to Wang et al. (2017). Briefly, 500 mg of frozen stool sample was homogenized after adding 3 ml of ultrapure water

and centrifuged at 12,000 x g for 10 min at 4°C. One milliliter of supernatant was homogenized with 0.2 mL 25% metaphosphoric acid and placed on ice for at least 30 min, then was centrifuged at 12,000 x g for 10 min at 4°C. Calibration curve was prepared to quantify the SCFAs using a commercial standard mixture of volatile fatty acids (CRM46975, Supelco, USA). Gas chromatograph determination was employed to quantify SCFAs content using a ThermoQuest TRACE 2000 (Milano, Italy). The chromatographic column specifications were WAX-DA 30 m × 0.25 mm, 0.5-µm film thickness. Using a splitless inlet, the injection volume and injector temperature were 1 µL and 220°C, respectively; the GC oven temperature programme was as follows: initial temperature 90°C for 0.50 min, then ramped to 150°C at 5°C/min and lasted 7 min; the temperature of the FID detector was at 230°C; the make-up flow, hydrogen flow and air flow were 30 mL/min, 40 mL/min and 400 mL/ min, respectively. The sample concentration was calculated referring to a commercial standard mixture of volatile fatty acids (CRM46975, Supelco, USA) and data processing was performed using Chrom-Card software (version 1.17).

2.6 Statistical analysis

XLSTAT Version 2016 statistical software was used for statistical analyses. The results are presented as the mean value ± standard deviation (SD) and analysed through a one-way ANOVA and Tukey post-hoc with significance at $p \leq 0.05$. Principal component analysis (PCA) was performed to process data from each animal in the *in vivo* study, in particular blood biochemical parameters, inflammatory and oxidative stress indices and SCFAs parameters. A score plot was made to shows the projection of individual animals in the multivariate space of two principal components. Furthermore, a biplot was made to interpolate the score of animals with the correlation matrix between variables and principal components (loading plot, data not shown) to understand which variables match with the different treatment groups.

3. Results and Discussion

3.1 Bioactive compounds and antioxidant capacity of Kavolì®

The freeze-dried Kavolì® water extract showed a total phenols content of 9.23±0.32 mg GAE/g dw, a flavonoids content of 4.71±0.30 mg CE/g dw and a flavonols content of 2.46±0.25 mg QE/g dw (Table 1). The antioxidant potential of Kavolì® extract was assessed through four *in vitro* assays concerning the antioxidant capacity (ORAC), the metal-related antioxidant power (FRAP) and the radical scavenging activity (DPPH and ABTS). Our results showed that *Brassica oleracea* leaves extract exhibited a strong antioxidant activity, with an ORAC value of 14.9 µmol TE/g dw, a FRAP value of 670 µM/g dw, a DPPH IC_{50} of 0.75 mg/mL and an ABTS IC_{50} of 0.29 mg/mL (Table 1).

These findings were similar or higher than those exhibited by other Brassicaceae studied previously using other extraction processes (Domínguez-Perles et al., 2010; Kaulmann et al, 2014; Bhandari and Kwak, 2015; Seong et al., 2016). The high levels of polyphenols and their flavonoid subgroups were likely responsible for the remarkable antioxidant capacities shown by Kavolì® extract, since it is known that phenolic content is strictly related to plant extracts antioxidant activity (Dai and Mumper, 2010). Therefore, dietary intake of some families of vegetables is usually associated to amelioration/prevention of oxidative stress-related diseases (Ksouri et al., 2012).

Table 1. Bioactive compounds and *in vitro* antioxidant activity of Kavolì®.

		Kavolì®
Bioactive compounds	Total phenols (mg GAE/g dw)	9.23±0.32
	Flavonids (mg CE/g dw)	4.71±0.30
	Flavonols (mg QE/g dw)	2.46±0.25
Antioxidant activity	ORAC (μmol TE/g dw)	14.89±1.17
	DPPH (IC_{50}=μg/ml)	752.02±53.86
	ABTS (IC_{50}=μg/ml)	286.05±24.83
	FRAP (μM/g dw)	669.96±39.31

Results are reported as means ± SD (n=3).

3.2 Quantification of polyphenols by HPLC-DAD

To get a more detailed picture of the polyphenols composition of Kavolì®, the main representatives and the estimate of their amount were determined by HPLC-DAD and are reported in Table 2. Chlorogenic acid and neochlorogenic acid were identified as the dominant components of both dry powder and water extract of Kavolì® (1097.7 and 975.3 mg/kg dw, in dry powder, and 2392.2 and 1084.7 mg/kg dw, in water extract, respectively). To follow, the other phenolic compounds in dry powder in order of quantity were protocatechuic acid, syringic acid, resveratrol, cynaroside, ellagic acid, trans sinapic acid, trans p-coumaric acid, trans ferulic acid, while rosmarinic acid was under the limit of detection (LOD). Differently, the other polyphenols detected in Kavolì® water extract were protocatechuic acid, trans sinapic acid, rosmarinic acid, trans ferulic acid, trans p-coumaric acid, while the other compounds were under the LOD. Although specific to the commercial product analysed, the present findings are similar to those reported by previous studies for different *Brassica oleracea* varieties. In fact, significant levels of chlorogenic acids and its isomers, neo- and crypto-chlorogenic acids as well as hydrocinnamic acids (ferulic, *p*-coumaric and sinapic acid) and protocatechuic acid have been previously reported as the principal polyphenols in leafy Brassica

species, such as kale, cabbage and Brussels sprouts, and have been identified as important predictors for their strong antioxidant capacity (Cartea et al. 2011; Kaulmann et al. 2014; Sharma et al., 2015). It is important to note that polyphenols content and their extraction efficiency are dependent on the polarity of the solvent used, since it determines the solubility of polyphenols based on their structure. Consequently, the reproducibility of these data is strictly dependent on the procedure used.

Table 2. Phenolic compounds, respective retention time (Rt), wavelengths (nm) and concentrations in dry powder and water extract of Kavolì® (mg/kg d.w.).

Phenolic compound	**Rt (min)**	**Wavelength (nm)**	**Dry powder (mg/kg dw)**	**Water extract (mg/kg dw)**
Chlorogenic acid	3.296	320	1097.7	2392.2
Neochlorogenic acid	2.722	320	975.3	1084.7
Protocatechuic acid	3.353	265	114.7	177.1
Syringic acid	5.154	320	57.39	≤LOD
Ellagic acid	6.710	265	35.67	≤LOD
Cynaroside	6.987	265	38.94	≤LOD
Trans p-coumaric acid	7.444	320	31.34	109.4
Trans sinapic acid	7.857	320	35.35	24.88
Trans ferulic acid	8.219	320	10.91	62.41
Rosmarinic acid	10.631	320	≤LOD	85.49
Resveratrol	13.766	320	41.64	≤LOD

LOD, limit of detection

3.3 The ameliorating effect of Kavolì® on plasma biochemical parameters

Differences in hepatotoxicity, lipid profile and cardiovascular risk indices among the three experimental groups were investigated by plasma analyses and are summarized in Table 3. In detail, HFD/STZ rats showed significant increases in plasma AST, ALT, BR, TG, TC, LDL-C, VLDL-C and glucose levels, in TC/HDL-C and LDL-C/HDL ratios, together with significant decreases in insulin level and AAI, in comparison to CTR group. These findings are in accordance to data previously reported for the same NAFLD model (Vornoli et al., 2014; Pozzo et al., 2016, 2019). The administration of 1 g/kg BW of Kavolì® for 5 weeks determined a significant improvement of most of the above mentioned indices. In fact, HFD/STZ+Kavolì® group showed significant decreases in AST, ALT, TG, TC, LDL-C, VLDL-C levels, in TC/HDL-C and LDL-C/HDL ratios, together with

increases, although not significant, in insulin level and AAI, in comparison to HFD/STZ group. No differences among the groups emerged regarding HDL-C, while BR was increased in all rats treated with HFD, confirming the presence of liver damage (Table 3). Other in vivo studies have shown improving effects due to *Brassicaceae* intake on many of the aforementioned blood parameters. Lee et al. (2009) showed that broccoli sprouts extract administration was able of lowering serum level of ALT, AST, TC, LDL-C, TG and AAI in rats fed a HFD. Similar effects were observed for *Brassica nigra* extract in Wistar rats with D-galactosamine-induced hepatotoxicity (Rajamurugan et al., 2012). *Brassica napus*, also called rapeseed, hydro-alcoholic extract significantly decreased plasma TC and LDL-C levels in diabetic rats (Akbari et al., 2016). Broccoli consumption reduced cholesterol serum levels (Kobayashi et al., 2002; Tomofuji et al., 2012) or plasma ALT concentration (Chen et al., 2016) in rats fed different types of HFD. Tuscan black cabbage (a variety of kale) sprout extract induced a significant decrement in various serum lipid parameters in HFD-treated rats (Melega et al., 2013). In a hyperlipidemic model of Wistar rats, the amelioration of serum lipid profile demonstrated the potential therapeutic power of another variety of Brassica oleracea: polyphenolic-rich red cabbage aqueous extract showed a beneficial effect against hypercholesterolemia and hypertriglyceridemia (Cruz et al., 2016). It is well known that perturbed values of such blood parameters represent an important risk factor for the development of various diseases. As other natural compounds of the family of *Brassicaceae*, Kavolì® proved to be useful in significantly improving blood parameter perturbations for slowing down or reverting the progression of cardiovascular disease and NAFLD. These beneficial properties are to be attributed mainly to the high content in polyphenols observed Kavolì® extract, such as chlorogenic and protocatechuic acids (Table 2), which have previously shown *in vivo* antioxidant power and cholesterol lowering ability by modulating the gene expression of PPAR-α (Wan et al., 2013; Grzelak-Błaszczyk at al., 2020).

Table 3. Biochemical parameters in plasma of all CTR, HFD/STZ and HFD/STZ+Kavolì® rats.

	CTR (n=5)	HFD/STZ (n=8)	HFD/STZ+Kavolì® (n=8)
AST (UI/l)	$93.98^{b}\pm5.45$	$198.38^{a}\pm42.86$	$91.06^{b}\pm16.86$
ALT (UI/l)	$39.06^{b}\pm9.02$	$143.17^{a}\pm27.79$	$69.95^{b}\pm9.56$
BR (mg/dl)	$0.86^{b}\pm0.08$	$1.00^{a}\pm0.06$	$1.01^{a}\pm0.06$
TG (mg/dl)	$75.73^{b}\pm5.71$	$186.26^{a}\pm38.21$	$137.56^{ab}\pm41.11$
TC (mg/dl)	$109.30^{b}\pm16.57$	$236.17^{a}\pm35.65$	$165.39^{b}\pm24.73$
HDL-C (mg/dl)	75.78±5.03	65.05±11.83	69.46±10.25
LDL-C (mg/dl)	$18.38^{b}\pm14.81$	$133.87^{a}\pm33.03$	$68.42^{b}\pm24.65$
VLDL-C (mg/dl)	$15.15^{b}\pm1.14$	$39.63^{a}\pm9.09$	$27.51^{ab}\pm8.22$

TC/HDL-C (ratio)	1.44^{b}±0.24	3.86^{a}±1.25	2.40^{ab}±0.29
LDL-C/HDL-C (ratio)	0.24^{b}±0.19	2.25^{a}±1.04	1.00^{ab}±0.39
Insulin (ug/l)	1.44^{a}±0.62	0.19^{b}±0.13	0.36^{b}±0.12
Glucose (mg/dl)	145.20^{b}±18.61	416.13^{a}±68.88	401.29^{a}±56.92
AAI*	294.36^{a}±128.88	41.87^{b}±16.30	74.34^{b}±13.81

Results are reported as means ± SD. Values within each row different letters (a,b) are significantly different ($p \leq 0.05$). *Antiatherogenic index (AAI) = HDL-C × 100/TC − HDL-C

3.4 Antioxidant and anti-inflammatory effects of Kavolì®

Compared to CTR rats, HFD/STZ treatment determined a decrease in GSH content, although not significant, and a statistically significant increase in carbonylated proteins and MDA levels in both liver and brain samples, in agreement with our earlier study in steatotic rats (Pozzo et al., 2019), as shown in Table 4. Such levels of oxidative stress in liver reflect an advanced stage of the steatotic disease, since the presence of reactive oxygen species due to mitochondrial dysfunction represents a critical moment occurring in the progression from NAFLD to NASH. All oxidative stress parameters were improved by the administration of Kavolì®; in particular, the intake of brassica extract by steatotic rats determined in both organs an increase in GSH content, although not significant, and a statistically significant decrease in carbonylated proteins and MDA levels (Table 4). Some previous studies have highlighted the protective effects of Nrf2 activation in reducing oxidative stress and nerve damage in both *in vitro* and *in vivo* models of neurodegenerative disorders (De Vries et al., 2008). Plant polyphenols have been linked to the improvement of HFD-induced liver oxidative stress due to their ability to activate Nrf2 transcription factor, which in turn is capable to increase the expression of antioxidant enzymes and GSH (Chuang & McIntosh, 2011; Tuzcu et al., 2017; Pozzo et al., 2019). In addition to polyphenols, other bioactive compounds that have been found particularly abundant in various species belonging to the *Brassicaceae* are glucosinolates (Fahey et al., 2001). Their degradation products, the isothiocyanates, are responsible for antioxidant effects similar to those attributed to polyphenols by the up-regulation of Nrf2-controlled antioxidant enzymes and influencing the activity of xenobiotic metabolism enzymes (Barillari et al., 2007; Canistro et al., 2012; Vivarelli et al., 2016). Saturated fatty acids intake has been associated also to an increased risk of diabetes-related neurodegenerative disease (Winocur and Greenwood, 2005). In light of this, Charradi et al. (2017) demonstrated that polyphenols-rich fruits are able to protect neurons from the oxidative stress damage due to HFD intake. Even when isolated, polyphenols, and flavonoids in particular, are able to improve HFD-induced oxidative stress both in liver and brain. In fact, past studies have shown that some polyphenols contained in Kavolì®, such as chlorogenic acid in liver, and trans-sinapic and

ferulic acid in brain, are able to improve or prevent oxidative stress in rats (Rodriguez de Sotillo and Hadley, 2002; Asano et al., 2017; Bais et al., 2018). In a study by Lee et al. (2017), chlorogenic and neochlorogenic acid have been identified as the major components of mulberry leaf extract promoting the lowering of hepatic lipid peroxidation and pro-apoptotic cascades inhibition in an ethanol-induced liver injury mouse model. Hao et al. (2015) demonstrated that chlorogenic acid attenuates the effects of oxidative brain damage through the maintenance of antioxidant homeostasis, the inhibition of lipid peroxidation and by supporting the endogenous antioxidant defence systems. Moreover, Semaming et al. (2015) showed that protocatechuic acid contributes to the prevention of brain oxidative stress in STZ-induced diabetic rats, and Güven et al. (2015) demonstrated that syringic acid treatment in cerebral ischemia reduces oxidative stress and neuronal degeneration. Herein, the improved liver and brain antioxidant defence observed in steatotic rats treated with Kavolì® are likely due to its high content in polyphenols (Table 4).

Concerning hepatic HO-1, an Nrf2 target, HFD/STZ rats showed a significant increase at both transcriptional and activity level, compared to controls (Figure 2). Nrf2 activation occurs only in later stages of the steatotic disease, thus suggesting that our model represent an advanced stage of NAFLD, as previously reported (Pozzo et al., 2016). Both *in vivo* and *in vitro* studies previously demonstrated that increased levels of HO-1 and/or HO activity are meant to interrupt progression of nutritional steatohepatitis by inducing an antioxidant pathway, suppressing production of cytokines, and modifying fatty acid turnover (Hinds et al.,2014; Yu et al., 2010). The treatment with Kavolì® resulted in a restore of the control values. This result needs to be further investigated since, as for the other Nrf2-mediated antioxidant responses, we would expect a further increase in HO-1 with respect to HFD/STZ values. Anyway, it is likely that in our model HO-1 increase occurs as an endogenous response to fatty liver disease, which is no longer needed after the health parameters improvement due to nutraceutical treatment.

In order to explore the potential ameliorative impact of Kavolì® on pathogenetic mechanisms involved in steatosis, the inflammatory genes TNFα and IL-6 were evaluated by quantitative RT-PCR. The HFD/STZ group showed a significant increase in the hepatic expression of the two genes, compared to CTR group, in accordance with what observed previously in our laboratories (Pozzo et al., 2016). One gram per kg BW Kavolì® treatment significantly decreased gene expression of both TNFα and IL-6, compared to HFD/STZ group (Figure 3). It is long known that liver inflammation and the related fibrotic process play a key role in the pathogenesis of NAFLD and its progression to NASH. HFD feeding leads to adipose tissue inflammation, determining the release of pro-inflammatory TNFα, which is responsible of the hepatic response to inflammation by secreting IL-6 and activating the fibrogenic stellate cells (Tilg et al., 2006). The administration of Kavolì® was able

to interrupt and revert this process likely due to its high content in polyphenols, whose anti-inflammatory capacity has been already proven by several studies in rats or mice (Yun et al., 2012; Shi et al., 2013; Niture et al., 2014; Oliveira Andrade et al., 2014).

Since both the extraction and preparation of the solution administered to rats were made in water, the antioxidant and anti-inflammatory properties shown by Kavolì® in the present study have to be attributed to water-soluble components, whose known properties has been widely discussed above.

Table 4. GSH content, malondialdehyde (MDA) and protein carbonylation in liver and brain of all CTR, HFD/STZ and HFD/STZ+Kavolì® rats.

		CTR (n=5)	HFD/STZ (n=8)	HFD/STZ+Kavolì® (n=8)
Liver				
	GSH (µmol/g)	2059.50±348.77	1260.77±444.05	1477.95±813.02
	MDA (nmol/g)	9.79^{b}±0.54	15.85^{a}±0.83	7.93^{c}±0.53
	Protein carbonylation (nmol/g)	397.89^{ab}±23.91	463.86^{a}±46.83	380.51^{b}±39.54
Brain				
	GSH (µmol/g)	243.65±47.85	201.41±53.18	253.24±77.00
	MDA (nmol/g)	2.32^{b}±0.20	5.64^{a}±0.53	1.78^{b}±0.09
	Protein carbonylation (nmol/g)	143.79^{b}±21.39	225.17^{a}±21.20	148.87^{b}±29.69

Results are reported as means ± SD. Values within each row different letters (a,b,c) are significantly different ($p \leq 0.05$).

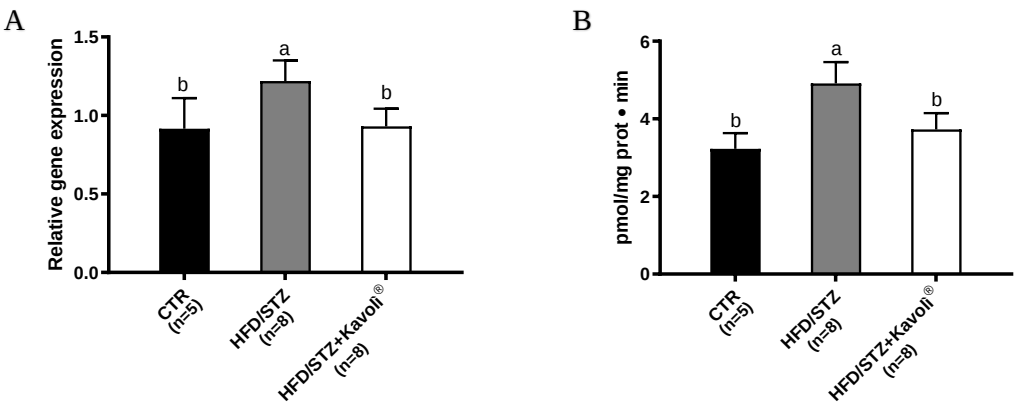


Figure 2. Hepatic HO-1 relative gene expression (A) and activity (B) expressed as pmol/mg protein

× min, measured in liver from all CTR, HFD/STZ and HFD/STZ+Kavolì® rats. Data represent the means ± SD (bars). Results of relative gene expression are normalized for the levels of housekeeping gene β-actin and referred to the mean of the controls, to which a value of 1 was assigned. a,b: values significantly different by one-way ANOVA-test, p≤0.05.

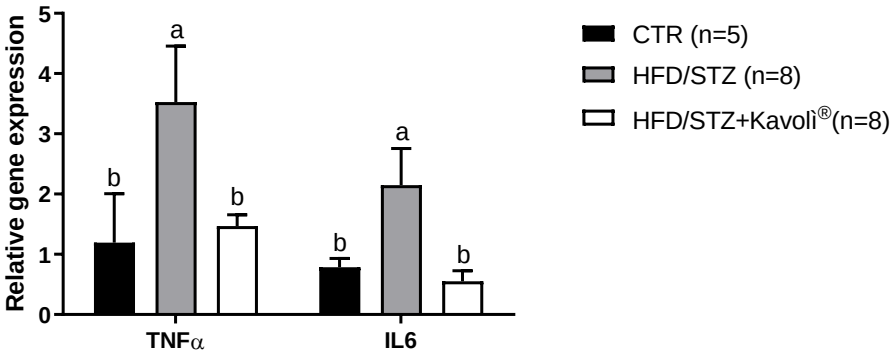


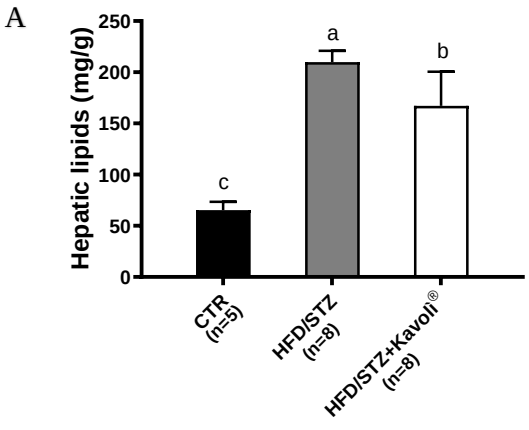
Figure 3. Relative gene expression of TNFα and IL6 measured by real-time RT-PCR in liver from all CTR, HFD/STZ and HFD/STZ+Kavolì® rats. Data represent the means ± SD (bars). Results are normalized for the levels of housekeeping gene b-actin and referred to the mean of the controls, to which a value of 1 was assigned. a,b: values significantly different by one way ANOVA-test, p≤0.05.

3.5. Lipid-lowering effect of Kavolì®

To verify the effectiveness of the treatment with Kavolì® in reducing hepatic steatosis, we measured the total hepatic lipid content in the three study groups. As expected, liver lipids resulted significantly higher in the HFD/STZ rats than in the controls, confirming the presence of steatosis. Five weeks of *Brassica oleracea* var. *acephala* administration improved the steatotic disease: while continuing to take HFD for the duration of the experiment, rats treated with Kavolì® showed a significant decrease in liver lipid content compared to HFD/STZ animals (figure 4A).

To confirm that the different treatments caused changes to the structure of liver tissue, histological analysis was performed using H&E staining. As shown by Figure 4B, sections from control animals exhibited normal liver architecture with prominent nucleus and well-preserved cytoplasm; the intact lobular structure with hepatic cell cords radiating outward from the central vein did not present any necrosis, inflammation or steatosis evidence (Figure 4B, panels a and d). In contrast, HFD/STZ rats showed severe changes in liver morphology, including extensive macro and micro-vesicular steatosis and infiltration of inflammatory cells, of probably lymphocytic nature, localized at the level of the central veins and the portal system (black arrow) (Figure 4B, panels b and e). As discussed above,

the establishment of the inflammatory process was confirmed also by the expression analysis of pro-inflammatory cytokines (Figure 2); TNF-α secretion mainly occurs by Kupffer cells, the activation of which is an event occurring in an advanced stage of liver steatosis. However, treatment with Kavolì® alleviated the degree of both liver steatosis and inflammatory cell infiltration (Figure 4B, panels c and f). The different histopathological features observed for the three experimental groups reflect also the above-illustrated values of blood biochemical parameters, such as blood transaminases and blood fats (Table 3). Some studies demonstrated that polyphenols-rich vegetables improve the hepatic lipid accumulation caused by HFD fed (Cheng et al., 2014). Moreover, it was also reported that the single isolated polyphenol can improve the liver lipids content due to a high fat diet administration, as in the case of chlorogenic acid (Wan et al., 2013; Rodriguez de Sotillo and Hadley, 2002). This same polyphenol in green coffee bean extract was found to be involved in the enhancement of fat metabolism in liver of mice (Shimoda et al., 2006). Recently, Yu et al. (2021) used an *in vitro* model of NAFLD for determining the molecular mechanisms behind the lipid-lowering ability of neochlorogenic acid, which represents also one of the major constituents of Kavolì®. Yu and collaborators showed that lipid accumulation in HepG2 cells was significantly improved by the down-regulation of the expression of SREBP1, which is responsible for unsaturated fatty acid biosynthesis. Since in our NAFLD model the expression of this transcription factor was up-regulated (Pozzo et al., 2016), the potential effect on SREBP1 pathway may represent one of the strategies for steatosis improvement by Kavolì® polyphenols; in particular, a major role is likely attributable to the two most representative ones of our extract (Table 2), given their above mentioned known effects.



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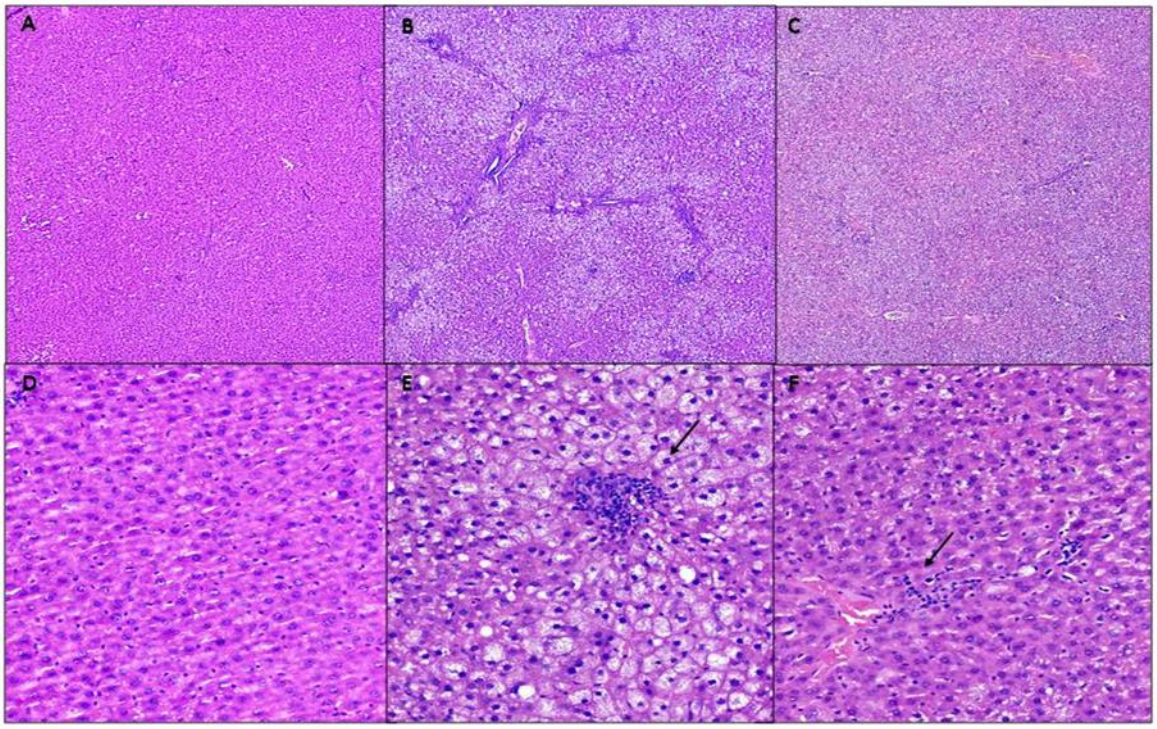

Figure 4. A: Total hepatic lipid content measured in liver tissue from all CTR, HFD/STZ and HFD/STZ+Kavolì® rats. Data represent the means ± SD (bars). a,b,c: values significantly different by one-way ANOVA-test, $p \leq 0.05$. B: Haematoxylin and eosin (H&O) staining of liver tissue from CTR, HFD/STZ and HFD/STZ+Kavolì® rats (a, b, c, respectively: magnification 4X; d, e, f: magnification 20X). Arrows indicates inflammatory infiltrates.

3.6 The effect of Kavolì® on SCFAs

In our study, acetate, propionate, isobutyrate and total SCFAs content resulted significantly increased in HFD/STZ rats compared to controls, whereas the treatment with Kavolì® determined a significant lowering of these values, both in plasma and feces. On the contrary, the significant HFD/STZ-induced decrease in butyrate level was significantly counteracted by Kavolì® administration in both the biological samples analyzed (Table 5). SCFAs are produced in intestinal lumen by microbiome fermentation of non-digestible carbohydrates, and are distinguished on the basis of carbon chain lengths: acetate (C2), propionate (C3), butyrate and isobutyrate (C4). The amount of these SCFAs, which account for about 95% of the total SCFAs, is strictly dependent from gut microbiota composition and metabolism, which in turn is influenced by the diet. It has been demonstrated that acetate and propionate are mainly produced by the phylum Bacteroidetes, whereas butyrate is the main product of the phylum Firmicutes (Hijova et al., 2007). A recent cross-sectional descriptive study by Gutiérrez-Díaz et al. (2021) linked macromolecular polyphenols intake to a direct

modulation of gut microbiota and, as a consequence, of SCFAs content in humans by enhancing the levels of relevant groups of intestinal bacteria, thus leading to proven beneficial health effects. The imbalance between SCFAs-producing bacteria have been previously linked to NAFLD. Our findings reflect those of Maciejewska et al. (2018) in which HFD administration determined an increase in the level of propionate, with the contemporary decrease in those of butyrate. The upward modulation in the levels of butyrate induced by the water extract of Kavolì® may have represented a key effect for NAFLD improvement in our rats. In fact, in a study by Endo and collaborators, the rise in butyrate levels by the action of butyrate-producing probiotics inhibited the progression of NAFLD through the gut-liver axis, in particular by the activation of the adenosine monophosphate-activated protein kinase (AMPK) and AKT signaling pathways, thus regulating lipid metabolism, oxidative stress and insulin sensitivity (Endo et al., 2013). In an *in vivo* study on mice, sinapine, the most representative polyphenol in *Brassica napus*, significantly affected the composition of intestinal microbiota, exerting beneficial effects on metabolic diseases such as NAFLD and diabetes (Li et al., 2019). Another mouse study confirmed the ability of Brassica species, and in particular of a fraction of *Brassica rapa* L, in altering the microbiota composition with the result of decreasing the numbers of Bacteroidetes and increasing those of butyrate-producing bacteria (Tanaka et al., 2016). In a very recent *in vivo* study in mice, Shi and collaborators demonstrated that chlorogenic acid is able to protect against HFD-induced hepatic steatosis and inflammation by regulating gut microbiota and increasing Glucagon-like peptide-1secretion, thus suggesting it could be used as a potential drug for prevention and treatment of NAFLD (Shi et al., 2021). This ability of chlorogenic acid in regulating gut microbiota was confirmed by other two *in vivo* study (Ye et al., 2021; Zhou et al., 2021). In the present experiment, the levels of SCFAs were brought to those of healthy control rats by the treatment with Kavolì®. For the above reasons, polyphenols of our Brassica water extract could be included among prebiotics based on the definition of non-digestible food ingredients that stimulate the growth of intestinal microbiota which are beneficial to the host.

Table 5. Short chain fatty acids in feces and plasma samples of all CTR, HFD/STZ and HFD/STZ+Kavolì® rats.

		CTR (n=5)	HFD/STZ (n=8)	HFD/STZ+Kavolì® (n=8)
Feces	Acetate (mmol/kg)	6.07^{b}±0.26	30.19^{a}±0.62	5.12^{b}±0.32
	Propionate (mmol/kg)	4.66^{b}±0.33	30.43^{a}±4.04	4.12^{b}±0.29
	Isobutyrate (mmol/kg)	0.33^{c}±0.03	8.79^{a}±0.41	5.17^{b}±0.21
	Butyrate (mmol/kg)	3.23^{a}±0.31	0.46^{c}±0.09	0.83^{b}±0.05
	Total SCFAs (mmol/kg)	14.28^{b}±0.67	69.78^{a}±3.67	15.24^{b}±0.64

Plasma	Acetate (µmol/l)	0.90^{c}±0.08	1.55^{a}±0.03	1.14^{b}±0.04
	Propionate (µmol/l)	0.10^{b}±0.02	0.27^{a}±0.02	0.15^{b}±0.03
	Isobutyrate (µmol/l)	0.02^{b}±0.01	0.06^{a}±0.02	0.01^{b}±0.00
	Butyrate (µmol/l)	0.05^{b}±0.00	0.08^{a}±0.01	0.03^{c}±0.01
	Total SCFAs (µmol/l)	1.07^{c}±0.11	1.96^{a}±0.03	1.33^{b}±0.08

Results are reported as means ± SD. Values within each row different letters (a,b,c) are significantly different ($p \leq 0.05$).

3.7 Principal component analysis (PCA)

To increase interpretability of datasets and better understand relationships among treatment groups, a PCA was performed. The first and second components (F1 and F2) explain respectively 63.36% and 11.28% of the total variance (74.63%). A score plot and a biplot depicting the scores of the samples is presented in Figure 5a, b. The score plot (a) shows that samples were clearly separated into three quadrants: one consisting of CTR, one of HFD/STZ and one of HFD/STZ+Kavolì® animals. PCA biplot (b) indicates how blood biochemical parameters, inflammatory and oxidative stress indices and SCFAs parameters contribute to the groups separation. As expected, HFD/STZ animals cluster with blood biochemical parameters of hepatotoxicity and blood lipids (AST, ALT, TG, TC, LDL-C), and oxidative stress and inflammatory markers (MDA, protein carbonylation of both liver and brain, and IL-6, TNF-α, HO-1 at transcriptional level). Furthermore, HFD/STZ rats cluster with acetate, propionate and isobutyrate, confirming the direct correlation between diet intake and gut microbiota composition. Consequently, CTR animals cluster with butyrate, but also with markers considered beneficial/with protective action (HDL-C, insulin, the antioxidant GSH and the AAI). The position of HFD/STZ+Kavolì® treatment group, closer to CTR animals and far from HFD/STZ, confirmed the differences with the latter group, indicating the efficacy of our test substance in ameliorating the values obtained for NAFLD animals and in restoring, as much as possible, those of CTR group (Fig. 5a, b). All in all, our PCA clearly showed the differences in the clustering of parameters taken into account in this study by distinguishing among three well defined groups.

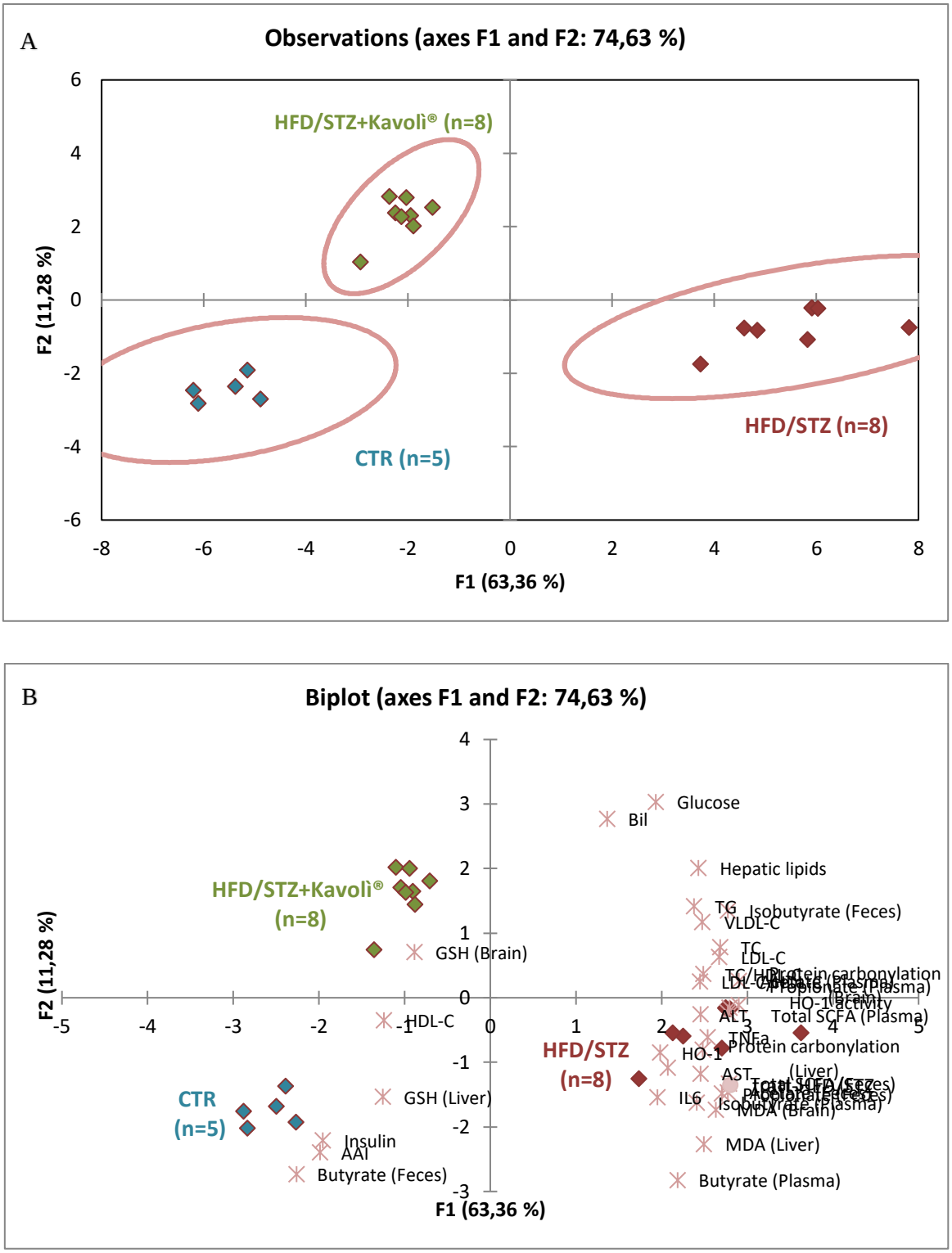

Figure 5. Principal component analysis (PCA) of effect of the groups treatment on the hepatic, blood, inflammatory and SCFAs content parameters: observation score plot (A) and biplot (B) of variables of the first two principal components (F1 and F2). Blue filled squares represent CTR, red filled squares HFD/STZ and green filled squares HFD/STZ+Kavolì® rats.

4. Conclusions

In the present study, a mixture of *Brassica oleracea* leaves, commercially known as Kavolì®, proved to be a bioactive phenolic compounds-rich food, able to effectively counteract the alterations associated to the HFD/STZ treatment in an *in vivo* model of NAFLD. The efficacy of the feed additive was also clearly validated through the PCA statistical tool. As observed in the present study, the consumption of polyphenol-rich foods such as brassicas is linked to several beneficial effects on health, including antioxidant, anti-inflammatory and lipid-lowering effect in animals. Overall, Kavolì® can be an important part of meal planning for patients with hyperlipidemia and steatosis. Nevertheless, further research is needed for deepen the mechanisms underlying these ameliorative effects on health, and the complex relationship between polyphenols and the intestinal microbiome, in light of the beneficial effect of Kavolì® polyphenols on SCFAs composition observed in our study.

Disclosure statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper. They also declare that their funding sources had no direct role in the study design, data collection, analysis and interpretation of the data, in the writing of the manuscript, or in the decision to publish the work.

Ethical approval

All animal procedures of the present experiment (Prot. n. 2612) were performed with the approval of the Local Ethical Committee and in accordance with the European Union Directive 2010/63/EU for animal experiments, and with the most restrictive Italian law (decreto legislativo 26/2014).

CRediT author statement

Andrea Vornoli, Luisa Pozzo: Writing- Original draft preparation, Conceptualization, Methodology, Investigation. **Teresa Grande:** Formal analysis. **Clara Maria Della Croce, Marco Matteucci, Laura Pucci, Morena Gabriele, Francesco Vizzarri, Marisa Palazzo, Július Árvay**: Investigation. **Moreno Paolini:** Writing- Reviewing and Editing. **Vincenzo Longo:** Conceptualization, Funding acquisition, Writing- Reviewing and Editing.

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