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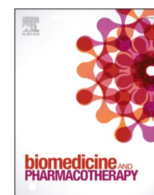

Original article

In vivo bioavailability and *in vitro* toxicological evaluation of the new butyric acid releaser N-(1-carbamoyl-2-phenyl-ethyl) butyramide

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ABSTRACT

A large body of evidence suggests that supplementation of butyric acid exerts beneficial intestinal and extra-intestinal effects. Unfortunately, unpleasant sensorial properties and unfavourable physico-chemical properties strongly limit its use in food supplements and foods for medicinal purposes. N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA) is a new butyric acid releaser in solid form with neutral sensorial properties. The aim of this investigation is to provide preliminary information on its pharmacokinetic and toxicological properties through the study of a) *in vivo* bioavailability of FBA administered by oral gavage to male and female Swiss CD1 mice in comparison with sodium butyrate, b) the influence of digestion on FBA stability through an *in vitro* simulated oro-gastro-duodenal digestion process, and c) *in vitro* toxicological profile by means of the Ames Test and Micronucleus Test. The results reveal that FBA is a good butyric acid releaser, being able to increase butyrate serum concentration in a dose and time dependent manner in both male and female mice with a pharmacokinetic profile similar to that obtained from sodium butyrate as such. These data are confirmed by investigating the influence of digestion on FBA, which undergoes extensive hydrolysis following oro-gastro-duodenal digestion, especially in duodenal conditions, with a residual concentration of less than 10% of the initial FBA concentration. Finally, in the Ames and Micronucleus Tests, FBA does not show any *in vitro* genotoxicity as it is non mutagenic in the Ames Test and results to be unable to induce chromosome breaks in the Micronucleus Test. In conclusion, FBA is a new butyric acid releaser that can overcome the disadvantages of butyric acid while maintaining the same pharmacokinetic properties and safety profile, as shown by the results of the preliminary *in vitro* toxicological studies performed in this investigation.

Abbreviations: 2AA, 2-amino-anthracene; 9AC, 9-amino-acridine-HCl; ATTC, American Type Culture Collection; BA, Butyric acid; BaP, Benzo-pyrene; BUNA, Sodium butyrate; CBPI, Cytokinesis-block proliferation index; CDL, Curved desolvation line; CHO, Chinese hamster ovary cells; ColC, Colchicine; CVS, Institutional Committee on the Ethics of Animal Experiments; CytB, Cytochrome B; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, Dimethyl sulfoxide; EFSA, European Food Safety Authority; FBA, N-(1-carbamoyl-2-phenyl-ethyl) butyramide; FOS, Fructooligosaccharides; GC-MS, Gas chromatography–mass spectrometry; GLP, Good laboratory practice; IL-β, Interleukin-beta; IL-2, Interleukin-2; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-12, Interleukin-12; LODs, Limits of detection; LOQs, Limits of quantification; MCT1, Monocarboxylate transporter isoform 1; MitC, Mitomycin C; MUC2, Mucin 2; NaAz, Sodium azide; 2NF, 2-nitro-fluorene; NF-κB, Nuclear factor kappa beta; NQO, 4-nitroquinoline-N-oxide; OECD, Organisation for Economic Cooperation and Development; RI, Replication Index; SGF, Simulated gastric fluid; SCFAs, Short chain fatty acids; SD, Standard deviation; SEM, Standard error of the mean; SIF, Simulated intestinal fluid; SIM, Single ion monitoring; SLC5A8, Sodium-coupled monocarboxylate transporter 1; SSF, Simulated salivary fluid; SOPs, Standard operating procedure; TNF-α, Tumor necrosis factor-alfa; UHPLC-ESI-MS, Ultra-performance liquid chromatography – electrospray ionization - mass spectrometer.

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1. Introduction

Butyric acid (C4:0, IUPAC name: butanoic acid, BA) is a short chain fatty acid (SCFA), naturally occurring in common foods such as butter, cheese, milk powder, creams, yoghurts and some bakery products [1]. BA is present in many foods, with butter being the most important dietary source, having with a maximum concentration of about 3 g/100 g [2,3]. The dietary intake of butyric acid is thus not sufficient to maintain gut health and to exert its numerous physiological benefits [1,2]. An important contributor towards an adequate concentration of butyric acid in the gut is provided by dietary non-digestible carbohydrates (*i.e.* pectin, resistant starch, and fructooligosaccharides - FOS), which are metabolized by colonic microorganisms to produce SCFAs (*i.e.* acetate, propionate and butyrate) [3–8] and other metabolites including water, methane, carbon dioxide [9]. Among SCFAs, butyrate has emerged as a potent modulator of several intestinal and extra-intestinal functions [10]. Anaerobic bacterial species from different genera including *Clostridium, Eubacterium, Butyrivibrio, Butyribacterium, Fusobacterium, Sarcina* and *Megasphaera* have been reported to produce butyrate [11]. The daily production of SCFAs in the colon of healthy humans is approximately 300–400 mmol, while physiological concentrations of butyric acid range from 1 to 10 mmol/L [12]. Butyrate is quickly absorbed in the colon *via* passive transport through non-ionic diffusion by SCFA/HCO^{3-} exchange, or active transport by SCFA transporters including monocarboxylate transporter isoform 1 (MCT1), coupled to a transmembrane H^+ gradient and Na^+ - coupled co-transporter SLC5A8 [8]. Butyrate uptake by colonic butyrate transporters is pH dependent, with 5.5 being the most optimal pH [13]. The butyrate absorbed in the distal colon is distributed to multiple organs [14]. Recent studies have shown an association between peripheral butyrate concentration and intake of dietary fiber, suggesting that butyrate is transported through the circulation, and peripheral organs may be affected by changes in its concentration [15].

Butyric acid is a bioactive molecule, modulating multiple therapeutic pathways [1,2]. Most of its mechanisms are associated with the regulation of gene expression through epigenetic mechanisms [16]. The intestinal effects of butyrate include the regulation of transepithelial ion transport, improvement of oxidative stress and inflammatory status of intestinal mucosa, modulation of intestinal motility, and prevention of colonic carcinoma [17]. Butyrate possesses anti-inflammatory activities in the intestinal epithelium by decreasing the production of pro-inflammatory cytokines (IL1β, IL2, IL6, IL8, IL12 and TNF-α) and downstream regulation of nuclear factor kappa beta (NF-κB) expression [18–20]. It modulates intestinal motility by stimulating the secretion of serotonin from enterochromaffin cells [21,22]. Butyrate also enhances the production of mucin, the main component of mucus, through upregulation of mucin genes MUC2, leading to increased intestinal protection against luminal agents [23]. Several studies have shown an inverse relationship between colonocyte DNA damage and large bowel butyrate availability, thus preventing the development and progression of colon cancers [24]. In addition, butyrate regulates fluid and electrolyte uptake, which makes it a potential agent of intervention in acute and chronic diarrhea [10,25–27]. The extra-intestinal effects of butyrate are numerous, including protective action against insulin resistance, obesity, hypercholesterolaemia and ischemic stroke [28–30].

In view of the large body of evidence suggesting the protective role of butyrate against several human conditions, oral supplementation of butyrate could be a promising approach in a large number of human diseases [10]. Moreover, enteral supplementation with butyrate was found to be safe and a better approach than the intake of SCFAs producing dietary fibers, which may cause abdominal distention, bloating and bacterial overgrowth in critically ill patients [31,32]. However, the clinical application of butyrate is still very limited because of its unfavorable organoleptic properties such as unpleasant taste and odor [11]. Butyrate formulations with different salts (Na^+, Ca^{++} and Mg^{++}) have been developed with better organoleptic properties, to ensure the effective use butyric acid in patients of all ages. Salt formulations have some advantages over free butyrate, as they are easier to handle owing to their less volatile properties and solid form [33,34]. Nevertheless, salt formulations show several disadvantages such as hygroscopic properties, poor water solubility, and deliquescence properties [35,36].

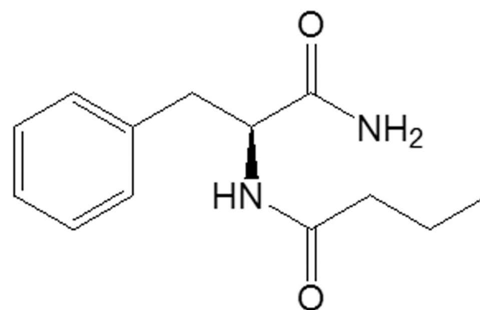

Fig. 1. N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA).

The need of new releasers of butyric acid thus prompted research into the development of new substances with better sensorial properties, to be assumed through the oral route especially for the pediatric population. N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA), is a patented new releaser of butyric acid which shows favorable organoleptic and physico-chemical properties (Fig. 1).

FBA, as a nutrient with an intentionally modified molecular structure, is classed as a novel food in Europe in accordance with European law (Regulation (EU) 2283/2015) [37], and therefore cannot be placed on the market or used in foods for human consumption until it is included in "the Union list" of novel foods authorised to be placed on the market within the European Union.

Thus, the aim of this investigation is to provide preliminary information of its pharmacokinetic and toxicological properties through the study of its *in vivo* bioavailability and *in vitro* toxicological profile. In addition, considering the data obtained from the *in vivo* bioavailability study, the influence of digestion on the stability of FBA was evaluated through an *in vitro* simulated oro-gastro-duodenal digestion process.

2. Materials and methods

2.1. Chemicals, bacterial strains and cells

Two industrial batches of FBA (N° UNF 5038b4I and N° UNF 5038b6I) were provided by ChiroBlock® (Wolfen, Germany). Sodium butyrate and butyric acid were purchased from Sigma- Aldrich (Milan, Italy). Phosphoric acid and ethyl acetate were provided by Carlo Erba (Milan, Italy).

All the compounds used for *in vitro* oral, gastric and duodenal digestion processes [38] have been reported as follows: potassium chloride (KCl), dihydrogen potassium phosphate (KH_2PO_4), sodium carbonate ($NaHCO_3$), magnesium chloride ($MgCl_2$), ammonium carbonate $(NH_4)_2CO_3$, calcium chloride ($CaCl_2$), sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH). All compounds were provided by Carlo Erba (Milan, Italy). Pancreatin from a porcine pancreas (extract of pig bile), α-amylase from *Bacillus licheniformis*, pepsin from porcine gastric mucosa and porcine bile extract, formic acid solution (1 M), water, methanol, acetonitrile LC-MS grade and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, Merck KGaA (Milan, Italy).

For the Ames Test, media and strains (*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* ECWP2UvrA), as recommended by OECD guideline 471 [39], were purchased from TrinovaBiochem (Giessen, Germany). Positive controls (99.9% pure as certified by the vendors), purchased from TrinovaBiochem, were sodium azide (NaAz) for strain TA1535; 2-amino-anthracene (2AA) for strains TA1535, TA1537 and ECWP2UvrA; 2-nitro-fluorene (2NF) for strains TA98 and TA100; benzo-pyrene (BaP) for strains TA98 and TA100;

4-nitroquinoline-N-oxide (NQO) for strain ECWP2UvrA; 9-amino-acridine-HCl (9AC) for strain TA1537. Metabolic activation was performed by a S9 fraction purchased from TrinovaBiochem.

As far as the *in vitro* mammalian cell Micronucleus Tests is concerned, CHO-K1 cells (code CCL-61™) were obtained from American Type Culture Collection (ATCC), (Manassas, VA, USA) and provided by LGC Standards (Milan, Italy). Mitomycin C (MitC) was purchased from Santa Cruz Biotechnology (California, USA). PBS and Triton X-100 were from Applichem GmbH (Darmstadt, Germany). 4′, 6-diamidino-2-phenylindole, (DAPI) was from Sigma Aldrich. Formaldehyde was from JTBaker (Deventer, The Netherlands). Colchicine (ColC) and Cytochalasin B were purchased from Sigma Aldrich (Milan, Italy). Ham's F-12K (Kaighn's) Medium, L-alanyl-L-glutamine (GlutaMAX™ Supplement), sodium bicarbonate, Penicillin-Streptomycin (10,000 U/mL), trypsin and EDTA were purchased from Thermo Fisher Scientific (Waltham, MA USA). Fetal bovine serum from Invitrogen was purchased from Thermo Fisher Scientific.

2.2. *In vivo bioavailability*

2.2.1. *Animals*

The experiments were performed on male and female Swiss CD1 mice (30–35 g) purchased from Charles Rivers (Calco, Lecco, Italy). They were housed in the animal care facility of the Department of Pharmacy, University of Naples "Federico II". Mice were acclimated to their environment for 1 week and food and water were made available *ad libitum*. Procedures involving animals and their care were conducted conforming with international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines, and the Basel declaration including the 3R concept). All procedures reported here were approved by the Institutional Committee on the Ethics of Animal Experiments (CVS) of the University of Naples Federico II and by the "Ministero della Salute" under protocol no. 851/2016. All efforts were made to minimize animal suffering, and at the end of all experiments the animals were euthanized by CO_2 overdose.

2.2.2. *Experimental procedures*

Butyrate serum concentration was evaluated after a single oral dose of FBA (20, 100, 200 mg/kg) and an equivalent dose of sodium butyrate (9.48, 47.42, 94.86 mg/kg), dissolved in 0.5 mL of water. More specifically, animals (n = 18 in total) were divided into 3 groups (each group containing 3 male and 3 female mice) and treated with low, medium and high doses. After overnight fasting, animals received FBA or sodium butyrate gavages, blood samples were obtained by cardiac puncture after 0, 10, 20, 30 and 60 min of gavage and kept at 4 °C.

2.2.3. *Extraction of butyric acid by mouse serum*

Each blood sample was centrifuged (2.500 rpm) for 12 min at 4 °C, obtaining serum. 0.2 mL of serum were acidified with 10 μL of 85% (w/v) phosphoric acid and mixed for 5 min at room temperature. Then, 0.2 mL of ethyl acetate was added to each sample and vortexed for 10 min, then centrifuged (14.000 rpm) for 45 min at room temperature. Finally, the supernatant (organic phase) was collected with a Pasteur pipette and placed in new glass tubes for gas-chromatography mass spectrometry (GC-MS) analysis.

2.2.4. *GC-MS analysis*

A GC (7890A; Agilent Technologies, Santa Clara, CA, USA) - MS (5977A MSD; Agilent Technologies) system with a GC column of 30 m (DB-WAX-UI, Agilent Technologies) internal diameter of 0.25 mm and film thickness of 0.25 μm was used. The GC was programmed according to the following run parameters: initial temperature of 50 °C, hold of 1 min, ramp of 10 °C min^{-1} up to a final temperature of 250 °C, total run time of 28.5 min, gas flow of 70 mL min^{-1}, splitless to maintain 12.67 p.s.i. column head pressure, and septum purge of 2.0 mL min^{-1}. Helium was the carrier gas (1.5 mL min^{-1}, constant). The parameters of the mass spectrometer used were a source at 230 °C and MS Quad at 150 °C. This was run in SIM mode. A GraphPad PRISM 5 program was used to determine serum butyrate concentration. To assist in quantifying FBA levels in serum samples, a calibration curve was generated to confirm the linear relationship between analyte peak area *versus* analyte concentration. Calibration curves (slope and intercept) and correlation coefficients (r) were calculated as regression parameters by linear regression. For this purpose, a calibration curve was prepared with a standard solution of butyric acid (1000 μg/mL, purity 99.7%) diluted to five final concentrations ranging from 0.01 to 1 μg/mL. The calibration curve was linear (y = 10.000.000x + 285.9421), with a correlation coefficient of 0.9988. Limits of detection (LODs) and quantification (LOQs) were calculated from the ratio between the standard deviation (SD) and the analytical curve slope, multiplied by 3 and 10 respectively, obtaining a LOD value of 5.21 μg/mL, and a LOQ value of 17.37 μg/mL.

2.3. *Effect of digestion process on FBA*

To verify the stability of FBA under digestion, a modification of the protocol by Minekus et al. [38] was applied.

2.3.1. *In vitro simulated oral digestion of FBA*

An aliquot of FBA (Batch N° UNF 5038b4I, 1.0 g precisely weighed) was dissolved in 0.7 mL of previously prepared simulated salivary fluid (SSF). The same procedure was followed for the blank sample using 5 mL of water instead of FBA solution. An aliquot of 0.1 mL (1500 U/mL) of fresh α-amylase solution prepared in SSF were added to both samples with 5 μL of $CaCl_2$ (0.3 M). Then, water was added to reach a final volume of 2 mL and the samples were incubated for 2 min at 37 °C. At the end of the oral digestion process, the samples were freeze dried and maintained at 4 °C prior to UHPLC-ESI-MS analysis.

2.3.2. *In vitro simulated gastric digestion of FBA*

An aliquot of FBA (Batch N° UNF 5038b4I, 1.0 g exactly weighed) was dissolved in 2 mL of bidistilled water for use as a sample. 1.5 mL of simulated gastric fluid (SGF) electrolyte stock solution was then added, followed by 0.32 mL porcine pepsin stock solution (25,000 U/mL prepared in SGF), 1 μL of $CaCl_2$ (0.3 M), 0.2 mL of HCl (1 M) to reach pH 3.0, and bidistilled water to obtain a total volume of 4 mL. The process was repeated, replacing the sample with a blank consisting of 2 mL of bidistilled water. Finally, the reaction vessels were placed onto a shaking platform at 37 °C for 2 h. At the end of the gastric digestion process, the samples were freeze dried and maintained at 4 °C prior to UHPLC-ESI-MS analysis.

2.3.3. *In vitro simulated duodenal digestion of FBA*

An aliquot of FBA (Batch N° UNF 5038b4I, 1.0 g exactly weighed) was dissolved in 4 mL of bidistilled water for use as a sample, mixed with 2.2 mL of simulated intestinal fluid (SIF) electrolyte stock solution, 1 mL of a pancreatin solution (800 U/ mL prepared in SIF electrolyte stock solution), 0.5 mL fresh bile (160 mM in fresh bile), 8 μL of $CaCl_2$ (0.3 M), 0.15 mL of NaOH (1 M) to reach pH 7.0, and bidistilled water to reach the final volume of 8 mL. The process was repeated for a blank of 4 mL of bidistilled water. Finally, the reaction vessels were placed onto a shaking platform at 37 °C for 2 h. At the end of the duodenal digestion process, the samples were freeze dried and maintained at 4 °C prior to UHPLC-ESI-MS analysis.

2.3.4. *In vitro simulated gastro-duodenal digestion process*

An aliquot of FBA (Batch N° UNF 5038b4I, 1.0 g exactly weighed) was dissolved in 2 mL of bidistilled water for use as a sample, and 0.3 mL of SGF electrolyte stock solution was then added, followed by 0.064 mL porcine pepsin stock solution (25,000 U/mL prepared in SGF), 1 μL of $CaCl_2$ (0.3 M), 0.2 mL of HCl (1 M) to reach pH 3.0 and bidistilled water to obtained a total volume of 4 mL. A 4 mL blank sample of bidistilled water was put through the same process. The reaction vessels were then

placed onto a shaking platform at 37 °C for 2 h. The simulated chyme samples were added to 2.2 mL of SIF electrolyte stock solution, 1 mL of a pancreatin solution (800 U/ mL prepared in SIF electrolyte stock solution), 0.5 mL fresh bile (160 mM in fresh bile), 8 μL of CaCl2 (0.3 M), 0.15 mL of NaOH (1 M) to reach pH 7.0, and bidistilled water to reach the final volume of 8 mL. Finally, the reaction vessels were placed onto a shaking platform at 37 °C for 2 h. At the end of the gastro-duodenal digestion process, the samples were freeze dried and maintained at 4 °C prior to UHPLC-ESI-MS analysis.

2.3.5. *In vitro simulated oro-gastro-duodenal digestion of FBA*

First, an aliquot of FBA (N° UNF 5038b4I, 1 g exactly weighed) was dissolved in 3.5 mL of previously prepared SSF. The same procedure was followed for the blank sample using 5 mL of bidistilled water. An aliquot of 0.5 mL (1500 U/mL) of fresh α-amylase solution prepared in SSF was added to each sample, with 25 μL of $CaCl_2$ (0.3 M). Then, water was added to reach a final volume of 10 mL and the samples were incubated for 2 min at 37 °C. At the end of the oral digestion process, the simulated bolus samples were added to 7.5 mL of SGF electrolyte stock solution, 1.6 mL porcine pepsin stock solution (25,000 U/mL prepared in SGF), 5 μL of CaCl2 (0.3 M), 1.0 mL of HCl (1 M) to reach pH 3.0 and bidistilled water to obtain a total volume of 20 mL. The reaction vessels were placed onto a shaking platform at 37 °C for 2 h. At the end of the oro-gastric digestion process, the simulated chyme samples were mixed with 11 mL of SIF electrolyte stock solution, 5 mL of a pancreatin solution (800 U/ mL prepared in SIF electrolyte stock solution), 2.5 mL fresh bile (160 mM in fresh bile), 40 μL of $CaCl_2$ (0.3 M), 0.75 mL of NaOH (1 M) to reach pH 7.0, and bidistilled water to reach the final volume of 40 mL. The reaction vessels were placed onto a shaking platform at 37 °C for 2 h. At the end of the oro-gastro-duodenal digestion process, the samples were freeze dried and maintained at 4 °C prior to UHPLC-ESI-MS analysis. To confirm the results obtained from FBA submitted to oro-gastro-duodenal digestion, this process was performed in triplicate on two industrial batches (Batch N° UNF 5038b4I and N° UNF 5038b6I), which were submitted to the whole digestion process and subsequent analysis.

2.3.6. *UHPLC-ESI-MS analysis*

Chromatographic analyses were performed using a UHPLC Nexera (Shimadzu, Milan, Italy) apparatus equipped with a LCMS-2010 single quadrupole coupled through an ESI source. UHPLC-ESI-MS data were acquired under positive ionization modes. The ion trap operated in Single Ionization Mode (SIM) *m/z* 235. To optimize the MS operating conditions, a preliminary experiment was performed: 10 μg/mL FBA (H_2O/MeOH: 50/50 with 0.1% formic acid) solutions were directly infused through the ESI interface at a flow rate of 25 μL/min into the mass spectrometer. Optimized conditions were as follows: CDL (Curved Desolvation Line) Temperature 250 °C, Nebulizing Gas Flow 1.5 L/min, Heat Block Temperature 300 °C.

Separation was achieved on a Kinetex Biphenyl (2.6 × 100 × 2.1 mm) column operating at 40 °C, protected by its corresponding guard column, both from Phenomenex, California, USA. A gradient elution was executed with acidified water (0.1% formic acid) as mobile phase A and acidified acetonitrile (0.1% formic acid) as mobile phase B, at a flow rate of 0.3 mL/min. The elution gradient for the separation of FBA involved moving from 10% B to 90% B over 2 min, and 90% B in isocratic mode for 1 min. The run time was 6 min in total, which includes the reconditioning of the column.

To assist in quantifying FBA levels in the digested samples, a calibration curve was generated to confirm the linear relationship between analyte peak area *versus* analyte concentration. Calibration curves (slope and intercept) and correlation coefficients (r) were calculated as regression parameters by linear regression. For this purpose, a calibration curve was prepared with a standard solution of FBA, diluted with the mobile phase to six final concentrations ranging from 1 to 150 μg/mL. The calibration curve was linear ($y = 0.000008x + 1.4206$), with a correlation coefficient above 0.998. The recovery, calculated at three spiked concentrations (low = 20 μg/mL, medium = 40 μg/mL, and high 80 μg/mL), was found to be 84.3 ± 0.23, 105.2 ± 0.12, and 97.3 ± 0.16%, respectively. Limits of detection (LODs) and quantification (LOQs) were calculated from the ratio between the standard deviation (SD) and the analytical curve slope, multiplied by 3 and 10 respectively, obtaining a LOD value of 1.18 μg/mL, and a LOQ value of 3.95 μg/mL.

The fully developed and validated method was used to determine residual concentration of FBA in the digested samples. In detail, aliquots of 10 mg of freeze-dried digested samples were dissolved in 2 mL of H_2O/MeOH: 50/50 prior to chromatographic analysis.

2.4. *In vitro toxicity tests*

Toxicological studies were performed in compliance with OECD principles of good laboratory practice (GLP), guidelines for testing of chemicals (OECD guideline 471 [39] and 487 [40]) and in accordance with the Standard Operating Procedures (SOPs) of the laboratories of Nutraceuticals at the Department of Pharmacy, University of Naples "Federico II".

2.4.1. *Ames Test*

A mutagenicity test was performed following the principles and procedures presented by OECD guideline 471 [39]. In brief, four cultures of *S. typhimurium* (strains TA98, TA100, TA1535, TA1537) and a culture of *E. coli* (strain ECWP2UvrA), were prepared from their main strain plates and used in their late exponential growth phase. Using the incorporation method, a negative control plate (with DMSO), positive control plates (with different chemicals depending on the bacterial strain), and FBA plates containing FBA solubilized into DMSO (used as solvent), were prepared at eight growing concentrations ranging from 0.0016 to 5 mg/plate (0.0016, 0.005, 0.016, 0.05, 0.16, 0.5, 1.6, and 5 mg/plate). In each tube, 0.1 mL of FBA solutions were added to 0.1 mL of fresh bacterial culture (containing approximately 10^8 viable cells), 0.5 sterile buffer, and 2.0 mL of overlay agar. The experiments were performed both in the presence and in the absence of metabolic activation (S9-mix), at a post-mitochondrial fraction concentration of 7% v/v. The contents of each tube were mixed and poured over the surface of a minimal agar plate. After solidification, the plates were incubated at 37 °C for 72 h. After the incubation period, the number of revertant colonies per plate was counted and their frequency was compared with that for the negative control group. Negative and positive control plates and FBA plates were tested in triplicate, the results expressed as number of revertant colonies per plate, and mean ± SD.

2.4.1.1. Acceptance of the test. Acceptance of the test was based on the following criteria: a) all experimental conditions requested by OECD guideline 471 were tested; b) considering FBA solubility, the criteria for the selection of FBA top test concentration (5 mg/plate) was consistent with those described in OECD guideline 471; c) none of the test concentrations exhibited a statistically significant increase in terms of number of revertants per plate compared with the concurrent negative controls; d) no concentration-related increase, nor any other trend, could be identified, e) all results were below the historical range of negative control data, f) all concurrent positive controls gave a statistically significant increases in terms of number of revertants compared with the concurrent negative controls.

2.4.2. *In vitro mammalian cell Micronucleus Tests*

A Micronucleus Tests was performed according to OECD guideline 487 [40].

2.4.2.1. Cells. Chinese Hamster Ovary K1 (CHO-K1) cells were chosen for micronucleus testing of FBA, in virtue of their extensive and

validated use in this specific test [41]. Cells were grown in Ham's F-12 Medium, containing 2 mM L-alanyl-L-glutamine, 1500 mg/L sodium bicarbonate and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin. Mother stocks were maintained in 75 cm^2 flasks (Corning, Tewksbury, MA, USA) in a cell culture incubator at 37 °C and with 5% CO_2 and 95% humidity. Duplication time of the CHO-K1 clone used in this set of experiments was of about 14 h, according to published cell characteristics [41]. The exponential growth phase was achieved by trypsinization using 0.25% (w/v) trypsin and 0.53 mM EDTA and by sub-cultivating at a 1:4 /1:8 ratio, with medium renewal once between cell splitting. CHO-K1 with passage numbers above 30 were not used for the assay. The absence of *Mycoplasma* contamination was confirmed before the beginning of each experiments by immunofluorescence identifying Mycoplasma DNA by DAPI staining.

2.4.2.2. FBA preparation and test condition. Since FBA is insoluble in water at room temperature, mother stocks (50 mg/mL) of FBA were prepared just prior to treatment by dissolving the solid test chemical in sterile DMSO, which is a well-established solvent for the Micronucleus Tests [41]. Test concentrations were obtained by diluting mother stock in complete cell culture medium. In a preliminary set of experiments, the insolubility of FBA at the test concentration of 3 mM was verified, with massive turbidity of the cell culture medium solution. This insolubility could not be rescued by increasing the DMSO content. As OECD guideline 487 includes the lowest concentrated turbid solution among test concentrations, a FBA 3 mM solution was therefore included. Six FBA concentrations were tested (3, 1, 0.3, 0.1, 0.03, and 0.01 mM) with the highest allowed DMSO concentration of 1% v/v. The pH of the FBA test solutions was checked and found to be 7.4. As expected in virtue of the chemical nature of FBA and the vehicle used to dissolve it, the osmolality of test dilutions was found to be 286 ± 22 mOSM/kg, and did not change compared to negative controls. FBA solutions with concentrations lower than 3 mM did not show signs of serum protein precipitation or other deleterious interaction between FBA and cell culture medium. Negative controls consisted of 1% v/v DMSO dissolved in cell culture medium. Positive controls for clastogenicity and aneugenicity consisted of MitC, (1.5 μg/mL, 4.5 mM), BaP (3 μg/mL, 12 mM) and ColC (1 μg/mL, 2.5 mM). When indicated, CytB 6 μg/mL was included in the assay.

2.4.2.3. Metabolic activation. With regards to the metabolic activation of FBA and BaP, this was achieved by exogenous metabolization using lyophilized S9 (S9-mix) previously supplemented with glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide and potassium chloride. S9-mix was reconstituted in deionized water and stored at − 80 °C. The activity of the S9-mix was tested by measuring its ability to activate BaP in the Ames Test. When indicated, S9-mix was used at a concentration of 2% (v/v) in the final test dilution.

2.4.2.4. Treatment schedules. The length of exposure to FBA was chosen to enable cell growth, chromosome damage and formation of micronuclei. The polyclone of CHO-K1 cells used showed a cell cycle length of ~14 h. Thus, the following treatment schedules were chosen:

- short treatment in the presence of metabolic activation: CHO-K1 were exposed to FBA and S9-mix for 6 h, then cultivated in the absence of FBA and in the presence of CytB for a further 28 h (2.0 normal cell cycle lengths after the beginning of treatment);
- short treatment in the absence of metabolic activation: CHO-K1 were exposed to FBA for 6 h to be then cultivated in the absence of FBA in the presence of CytB for a further 28 h;
- long treatment in the absence of metabolic activation: CHO-K1 were continuously exposed to FBA for 34 h in the presence of 6 μg/mL CytB.

Since any sign of cell detachment could be detected during the washing-out of FBA, it was not necessary to recover cells from conditioned media.

2.4.2.5. Micronucleus Test procedure. For evaluation of cytotoxicity, CHO-K1 cells were propagated from stock cultures and seeded into 25 cm^2 culture flasks with 5 mL of a (~ 0.6–0.7) × 10^5 cells/mL stock and incubated for 16 h. This cell density value was chosen so that monolayers would continue to grow exponentially until harvest time, and would not reach confluence. Before adding FBA solutions, a cell count was taken from two specified flasks. Medium was then replaced with FBA solutions according to the treating schedules. Negative controls (cells treated with vehicle) and positive controls (cells treated with MitC, BaP and ColC) were also processed in the same way as FBA treated cultures. Upon the 28 h time point, cells were harvested by trypsinization and counted by using the automatic Cell counter Luna (Logos Biosystem, South Korea). Measurement of cell proliferation was performed to assure that sufficient treated cells underwent mitosis during the test.

For micronucleus induction, parallel cultures of CHO-K1 cells were seeded in 6-well slide chambers with 3 mL of cell suspension, resulting in a plating density of ~50,000 cells/cm^2, and incubated for 16 h. Medium was then replaced with FBA solutions according to the treating schedules. Negative controls consisted of solvent diluted in the culture medium. Positive controls were processed in the same way as the FBA treated cultures. Upon 28 h of incubation, the medium was removed and cells were rinsed once with 1 mL of PBS and fixed in paraformaldehyde 3.7% for 30 min. The wells were then washed twice in PBS 1X, permeabilized in 0.1% Triton X100 diluted in PBS (10 min) and stained with DAPI (30 μM). DAPI fluorescence was measured using following parameters: λ excitation 351 nm, λ emission 450 nm, using an IRIS fluorescent microscope (Logos Biosystem, South Korea).

Cells were analyzed manually for the presence of micronuclei using the criteria developed by Eastmond and Tucker [42]. Binucleate cells with irregular shapes or where the two nuclei differed greatly in size were excluded. Poorly spread multi-nucleate cells were excluded. Cells containing more than two main nuclei were not analysed for micronuclei.

The cytokinesis-block proliferation index (CBPI) and the Replication Index (RI) were measured to estimate the cytostatic activity of each treatment by comparing values in the treated and control cultures.

CBPI = (N° mononucleate cells + 2 x N° binucleate cells + 3 x N° multinucleate cells)/(Total number of cells).

RI = ((N° binucleated cells + 2x N° multinucleate cells)/total number of cells in treated cultures)/((N° binucleated cells + 2x N° multinucleate cells)/total number of cells in control cultures) X 100.

Cell counting verified duplication of cells in culture during or following treatment with FBA as well as cytotoxicity of the test compounds.

% Citotoxicity = 100–100*(((Total number of cells at the end of the assay-Total number of cells plated at time 0)treated)/((Total number of cells at the end of the assay-Total number of cells plated at time 0) untreated)).

The historical negative values were obtained by averaging more than 20 experiments and resulted in a percentage of micronuclei in binucleated cells upon treatment with vehicles of 0.30 ± 0.2 (range 0.2–1; 95% CI [0.25–0.36]) and of 0.4 ± 0.3 (range 0.2–1.1; 95% CI [0.30–0.50]), in the presence of Cyt B and in the absence of Cyt B, respectively.

The historical negative values for 36 h incubation with Cyt B in the absence of S9-mix was 0.29 ± 0.20 (range 0.2–1.1; 95% CI [0.2–0.35]). The historical positive values were obtained by averaging more than 20 experiments and resulted in a percentage of micronuclei in binucleated cells of 1.2 ± 0.3% (range 1.2–1.7; 95% CI [1.1–1.3]) upon treatment with MitC in the absence of Cyt B, 2.0 ± 0.4% (range 2–3.1; 95% CI [1.8–2.2]) for ColC in the absence of Cyt B, and of 1.7 ± 0.3% (range 1.5–3; 95% CI [1.6–1.8])for BaP in the presence of Cyt B.

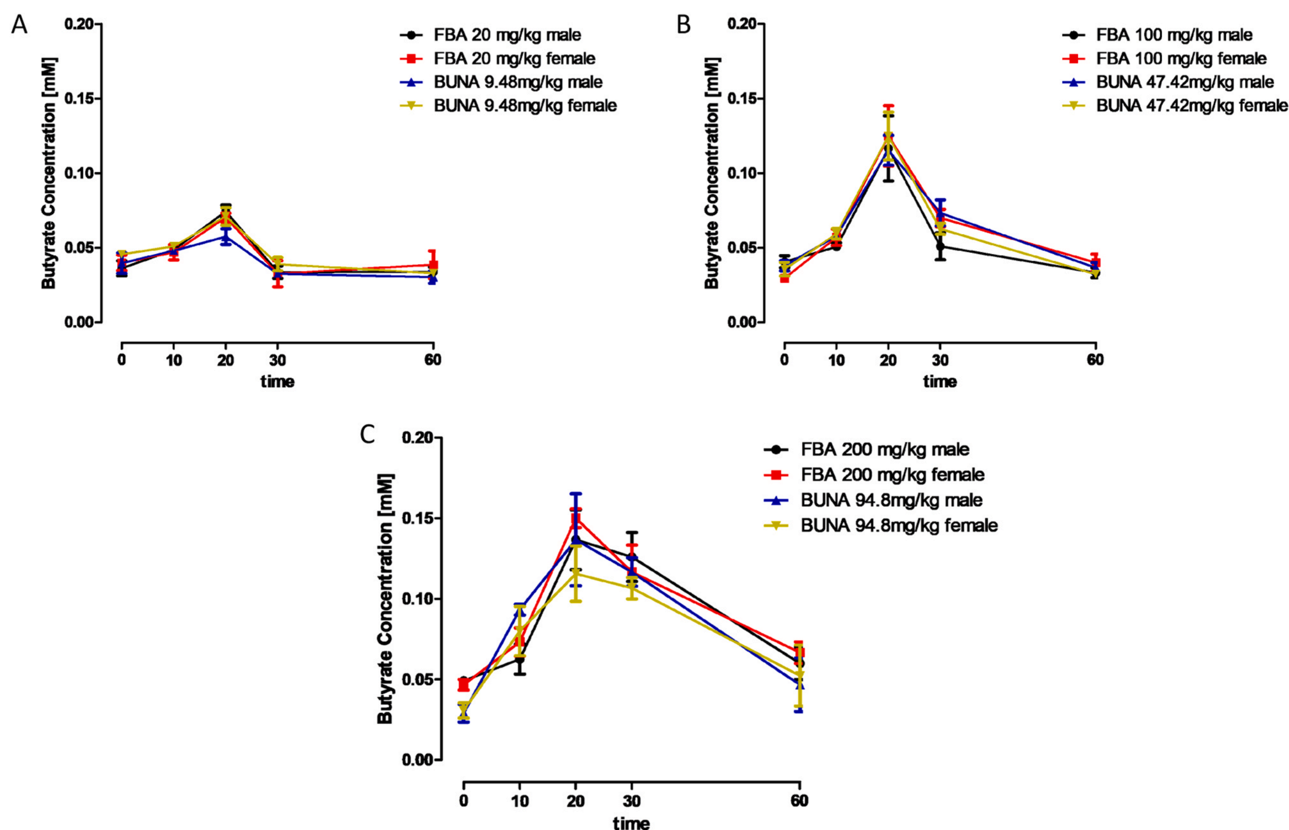

Fig. 2. Butyric acid serum concentration after single oral administration of FBA (male -black square- and female -red square-) and sodium butyrate (BUNA) (male -blue triangle- and female -yellow triangle-). (A) low FBA (20 mg/kg), and sodium butyrate (9.48 mg/kg) doses; (B) medium FBA (100 mg/kg) and sodium butyrate (47.42 mg/kg) doses; (C) high FBA (200 mg/kg), and sodium butyrate (94.86 mg/kg) doses. For all groups, data are shown as mean ± SEM (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4.2.6. Acceptance of the test. Acceptance of the test was based on the following criteria: a) all experimental conditions requested by OECD guideline 487 were tested; b) more than 10000 cells were scored and six FBA concentrations were tested; c) considering FBA solubility, the criteria for the selection of FBA top test dilution (FBA 3 mM) was consistent with those described in OECD guideline 487; d) the results obtained from the negative control were consistent with the laboratory's historical negative control database; e) concurrent positive controls induced responses that are compatible with those generated in the laboratory's historical positive control database and produced a statistically significant increase compared with the concurrent negative control; f) as shown by the reported CBPI and RI values, cell proliferation criteria in the solvent control, in the positive controls and in the test dilutions of FBA were all fulfilled.

3. Results

3.1. Comparison of in vivo bioavailability of FBA and butyric acid

To determine the butyrate concentrations before and after the administration of increasing concentrations of FBA and equivalent doses of sodium butyrate by oral gavages, a GC-MS method was developed. The butyrate concentrations determined in mouse serum showed that no significant differences were observed between male and female mice for all doses (20, 100, and 200 mg/kg for FBA and 9.48, 47.42, and 94.86 mg/kg for sodium butyrate), and at all experimental times (0, 10, 20, 30, 60 min). Total butyrate concentration observed in mice serum samples ranged from 0.030 to 1.137 mM.

As reported in Fig. 2A, the lowest dose of FBA (20 mg/kg) and the equivalent lowest sodium butyrate dose (9.48 mg/kg) induced little increase in serum butyrate concentration, and only at 20 min after oral administration a significant increase in serum concentrations was registered (percent increase corresponding to 47.3% and 31.3%, of the initial concentration, respectively). Oral administration of FBA at 100 mg/kg induced a slight increase in serum butyrate concentration after 10 min and a marked increase after 20 min. It is noteworthy that similar profiles were obtained for the equivalent dose of sodium butyrate (47.42 mg/kg) (35.2% and 36.2%; 70.1% and 69.2%, respectively; Fig. 2B). Finally, the highest tested doses (200 mg/kg of FBA and 94,86 mg/kg of sodium butyrate) induced a marked increase in serum butyrate concentration after 10 min (29,5% and 65,1%, respectively), with maximum serum concentration attained after 20 min (66,4% and 76,2%, respectively), remaining high at the 30 min time (60,4% and 73,2%, respectively). Finally, at the time of 60 min, butyrate serum concentration by FBA oral administration showed a slight increase compared to the sodium butyrate group (Fig. 2C).

3.2. Influence of in vitro simulated oro-gastro-duodenal digestion processes on FBA stability

The similar profiles found in butyrate mouse serum concentrations after the administration of FBA and sodium butyrate may suggest that FBA releases butyric acid in the intestinal tract, before the absorption, then both the butyrate released by FBA and the butyrate administered as such are absorbed without differences in absorption kinetics. Thus, to evaluate the influence of digestion on FBA and the possible residual concentration of FBA following digestion, an *in vitro* simulated digestion protocol [38] was applied, using conditions and composition of digestive fluids broadly held in consensus as physiologically relevant. To determine the FBA concentration before and after digestion, a

Table 1
FBA concentrations before and after oral, gastric, duodenal, gastro-duodenal and oro-gastro-duodenal digestion and concentration loss percentages.

In vitro simulated digestion	FBA concentration before digestion (g/ 10 mL)	FBA concentration after digestion (g/ 10 mL)	FBA concentration loss (%)
Oral digestion	5.40	5.30 ± 0.100	1.8
Gastric digestion	5.15	2.15 ± 0.150	41.7
Duodenal digestion	5.25	0.50 ± 0.005	90.5
Gastro-duodenal digestion	5.50	0.45 ± 0.005	91.8
Oro-gastro-duodenal digestion	5.35	0.50 ± 0.002	90.7

UHPLC-ESI-MS method was developed and validated. The results (Table 1) show that after oral digestion, FBA concentration loss percentage is under 2%. On the contrary, after the gastric digestion process, FBA underwent significant degradation with a FBA concentration loss percentage of 41.7%. After duodenal, gastro-duodenal and oro-gastro-duodenal processes, FBA underwent almost total degradation, with a FBA concentration loss percentage above 90%.

The residual concentration of FBA was under 10% after the whole digestion process, as confirmed by the results obtained from additional experiments performed in triplicate, in which two FBA batches were submitted to the oro-gastro-duodenal process. The results (Table 2) confirm that the residual FBA concentration ranges from 5.4% to 9.3%.

3.3. *In vitro toxicity of FBA*

3.3.1. *Ames Test*

To detect possible point mutations (*i.e.* substitution, addition or deletion of one or a few DNA base pairs) induced by FBA, the bacterial reverse mutation test was applied using four different *S. typhimurium* strains (TA98, TA100, TA1535 and TA1537) and an *E. coli* strain (WP2 urA), each representing a specific type of mutation caused by a substance. The genetic backgrounds of the used bacterial strains were controlled simultaneously to the test experiments (data not shown). Then, to confirm the sensitivity of the test system and the activity of the S9-mix, a positive control was set up for each bacterial strain, showing significant increases in the number of revertant colonies in line with historical laboratory data.

The results reported in Table 3 showed that exposure of the bacterial strains to FBA at eight increasing concentrations (ranging from 0.0016 to 5 mg/plate) did not increase the number of revertant colonies in any strain, at any of the tested concentrations, either in the presence or absence of S9-mix, in comparison with the negative control (DMSO, which is used as the FBA solvent in this test). Based on these results, FBA resulted to be non mutagenic under the conditions tested.

3.3.2. *In vitro mammalian cell Micronucleus Test*

The aim of the *in vitro* Micronucleus Test was to evaluate the ability for FBA ability to induce the formation of small membrane-bound DNA fragments, such as micronuclei in the cytoplasm of the interphase cell.

The results are reported in Tables 4 and 5. According to OECD guideline 487, the optimum maximum concentration for micronucleus analysis is defined as the concentration inducing around 50–60% toxicity. The maximum concentration tested for FBA was 3 mM (1% v/v DMSO). We tested six decreasing concentrations, namely 3, 1, 0.3, 0.1, 0.03, and 0.01 mM. Target toxicity was not achieved for any of the tested concentrations. Upon 6 h of incubation with FBA (both in the presence and in the absence of S9-mix), RI and CBPI did not show signs of cytostasis at any of the tested concentrations of FBA. Cell counting revealed minimal cytotoxicity at the 3 mM FBA test concentration, which induced the death of 6% of the cell culture. Upon 34 h of incubation with FBA (both in the presence and in the absence of S9-mix), RI and CBPI did not show signs of cytostasis at any of the tested FBA concentrations. Cell counting revealed minimal cytotoxicity at the 3 mM FBA test concentration, which induced the death of 12% of the cell culture. The frequencies of micronucleated cells were similar for all treatment regimens and concentrations of FBA, and none were significantly ($p > 0.05$) higher than those observed in the concurrent vehicle controls (Tables 4 and 5).

4. Discussion

A large body of scientific evidence supports butyric acid supplementation for its beneficial and protective effects at gastrointestinal and systemic levels. Nevertheless, the use of butyric acid as an ingredient of food supplements or foods for medical purposes is limited due to its very unpleasant.

sensorial and unfavourable physico-chemical properties. N-(1-carbamoyl-2-phenyl-ethyl) butyramide is a new butyric acid releaser in solid form and with neutral sensorial properties. FBA, as a nutrient with an intentionally modified molecular structure, in Europe is a novel food according to European legislation, which may become a new source of butyric acid only after its inclusion in the Union list of accepted novel foods.

Thus, in this preliminary investigation, the *in vivo* bioavailability in experimental animals and the *in vitro* toxicology of FBA were assessed, using a test for gene mutation in bacteria (Ames Test) and a cytogenetic test for chromosomal damage (*in vitro* Micronucleus Test).

As far as *in vivo* bioavailability is concerned, acute oral administration of sodium butyrate yielded a rapid increase in serum concentration at the medium and particularly at the highest doses used. Similarly, oral FBA produced a minor increase in butyrate serum concentration after 10 and 20 min at medium dose, this characteristic was more relevant at the highest dose and endured for a longer time, up to about 60 min. These data suggest that FBA is a good butyrate releaser and that high doses of this new compound ensure both rapid and prolonged release.

The results obtained from the *in vivo* bioavailability study prompted us to determine the residual concentration of FBA in the gastrointestinal tract after the digestion process. Considering that FBA is a secondary amide that undergoes hydrolysis in both acidic and alkaline environments, the FBA undergoes hydrolysis in acidic gastric conditions as expected, catalyzed by the proteolytic enzymes to produce butyric acid, then undergoing extensive hydrolysis in duodenal conditions where it is almost totally hydrolyzed in the presence of trypsin, with a residual concentration of under 10% of FBA concentration before digestion.

Table 2
FBA batch number, content before and after oro-gastro-duodenal digestion and loss percentages.

FBA Batch number	FBA before digestion (g)	FBA after digestion (g)	FBA loss percentage (%)	FBA loss percentage mean % ± SD
UNF 5038b4I_01	1.0091	0.161	84	90.7 ± 5.89
UNF 5038b4I_02	0.9871	0.067	93	
UNF 5038b4I_03	1.008	0.050	95	
UNF 5038b6I_01	0.9658	0.053	94	94.6 ± 0.92
UNF 5038b6I_02	1.0029	0.056	94	
UNF 5038b6I_03	1.0334	0.041	96	

Table 3
Number of revertants⁄plate for: A) S. typhimurium TA98 strain, B) S. typhimurium TA100 strain, C) S. typhimurium TA1535 strain, D) S. typhimurium TA1537 strain and E) E.coli WP2 urA in the presence and absence of metabolic activation (S9-mix) of FBA at different concentrations, DMSO, and positive controls.

A) (mg/plate)	−S9-mix				+S9-mix			
	n1	n2	n3	Mean ± SD	n1	n2	n3	Mean ± SD
Negative Control	25	29	28	27 ± 2	27	42	45	38 ± 10
FBA: 0.0016	32	37	34	34 ± 3	44	48	52	48 ± 4
FBA: 0.0050	30	32	35	32 ± 3	35	39	39	38 ± 2
FBA: 0.0160	21	23	21	22 ± 1	38	40	42	40 ± 2
FBA: 0.0500	27	34	33	31 ± 4	46	51	52	50 ± 3
FBA: 0.1600	27	31	30	29 ± 2	42	48	43	44 ± 3
FBA: 0.5000	25	27	28	27 ± 2	38	43	45	42 ± 4
FBA: 1.6000	35	38	36	36 ± 2	40	42	45	42 ± 3
FBA: 5.0000	34	39	39	37 ± 3	40	43	41	41 ± 2
Positive Control	1201	1225	1216	**1214 ± 12**	1320	1332	1330	**1327 ± 6**

Negative Control: dimethyl sulfoxide (DMSO—100 μL/plate); Positive Control: 2-Nitrofluorene (2.0 μg/plate) in the absence of S9-mix and Benzo(a)pyrene (6 μg/plate) in the presence of S9-mix.Historical negative in the absence of S9- mix: Range 22–57 (mean ± SD = 29 ± 8). Historical negative in the presence of S9- mix: Range 20–53 (mean ± SD = 43 ± 13). Historical positive in the absence of S9- mix: Range 1109–1363 (mean ± SD = 1214 ± 46). Historical negative in the presence of S9- mix: 1 Range 236–1348 (mean ± SD = 1321 ± 33).

B) Dose (mg/plate)	−S9-mix				+S9-mix			
	n1	n2	n3	Mean ± SD	n1	n2	n3	Mean ± SD
Negative Control	173	181	166	173 ± 87	190	215	222	209 ± 17
FBA: 0.0016	187	198	166	184 ± 16	206	218	227	217 ± 11
FBA: 0.0050	200	212	188	200 ± 12	220	230	240	230 ± 10
FBA: 0.0160	190	205	175	190 ± 15	210	249	221	227 ± 20
FBA: 0.0500	210	227	193	210 ± 17	230	217	240	229 ± 12
FBA: 0.1600	196	214	178	196 ± 18	196	232	242	223 ± 24
FBA: 0.5000	195	207	183	195 ± 12	215	216	222	218 ± 4
FBA: 1.6000	184	200	168	184 ± 16	204	249	223	225 ± 23
FBA: 5.0000	215	229	201	215 ± 14	225	223	230	226 ± 4
Positive Control	1542	1575	1510	**1542 ± 33**	1721	1733	1728	**1727 ± 6**

Negative Control: DMSO (100 μL/plate); Positive Control Sodium Azide (1.25 μg/plate) in the absence of S9-mix and Benzo(a)pyrene (6 μg/plate) in the presence of S9-mix. Historical negative in the absence of S9-mix: Range 144–240 (mean ± SD = 195 ± 15). Historical negative in the presence of S9-mix: Range 176–250 (mean ± SD = 211 ± 21). Historical positive in the absence of S9-mix: Range 1428–1620 (mean ± SD = 1480 ± 80). Historical positive in the presence of S9-mix: Range 1600–1923 (mean ± SD = 1693 ± 72).

C) Dose (mg/plate)	−S9-mix				+S9-mix			
	n1	n2	n3	Mean ± SD	n1	n2	n3	Mean ± SD
Negative Control	15	17	19	17 ± 2	20	21	14	18 ± 4
0.0016	18	15	21	18 ± 3	24	27	18	23 ± 5
0.0050	18	16	20	18 ± 2	21	25	29	29 ± 4
0.0160	17	18	21	19 ± 2	26	32	33	33 ± 4
0.0500	29	27	29	28 ± 1	29	26	22	26 ± 4
0.1600	17	19	21	19 ± 2	19	19	24	21 ± 3
0.5000	21	23	22	22 ± 1	19	25	29	24 ± 5
1.6000	29	33	26	29 ± 4	28	24	29	27 ± 3
5.0000	31	33	35	33 ± 2	21	26	29	25 ± 4
Positive Control	201	221	217	**213 ± 11**	220	229	201	**217 ± 14**

Negative Control: DMSO (100 μL/plate); Positive Control: Sodium Azide (1.25 μg/plate) in the absence of S9-mix and 2-aminoanthracene (2 μg/plate) in the presence of S9-mix.Historical negative in the absence of S9-mix: Range 12–45 (mean ± SD = 26 ± 5). Historical negative in the presence of S9-mix: Range 17–35 (mean ± SD = 23 ± 9). Historical positive in the absence of S9-mix: Range 195–240 (mean ± SD = 202 ± 36). Historical positive in the presence of S9-mix: Range 222–286 (mean ± SD = 232 ± 26).

(*continued on next page*)

Table 3 (*continued*)

D) Dose (mg/plate)	−S9-mix				+S9-mix			
	n1	n2	n3	Mean ± SD	n1	n2	n3	Mean ± SD
Negative Control	20	23	26	23 ± 3	22	26	18	22 ± 4
0.0016	26	22	18	22 ± 4	29	29	25	28 ± 2
0.0050	27	27	25	26 ± 1	28	28	22	29 ± 3
0.0160	28	26	28	27 ± 1	34	27	20	33 ± 7
0.0500	32	30	32	31 ± 3	23	26	29	26 ± 3
0.1600	28	34	32	31 ± 4	25	21	21	22 ± 2
0.5000	33	28	35	32 ± 4	36	30	24	30 ± 6
1.6000	32	37	30	33 ± 4	29	29	33	30 ± 2
5.0000	33	37	39	36 ± 3	23	35	26	28 ± 6
Positive Control	200	207	193	**200 ± 7**	220	235	223	**226 ± 8**

Negative Control: DMSO (100 µL/plate); Positive Control: 9-aminoacridine HCl (50.0 µg/plate) in the absence of S9-mix and 2-aminoanthracene (2 µg/plate) in the presence of S9-mix.Historical negative in the absence of S9-mix: Range 19–40 (mean ± SD = 32 ± 10). Historical negative in the presence of S9-mix: Range 18–43 (mean ± SD = 31 ± 6). Historical positive in the absence of S9-mix: Range 187–250 (mean ± SD = 210 ± 42). Historical positive in the presence of S9-mix: Range 223–270 (mean ± SD = 216 ± 34).

E) Dose (mg/plate)	−S9-mix				+S9-mix			
	n1	n2	n3	Mean ± SD	n1	n2	n3	Mean ± SD
Negative Control	36	40	32	36 ± 4	40	44	46	43 ± 3
0.0016	33	37	35	35 ± 2	47	47	44	46 ± 2
0.0050	27	34	38	33 ± 6	49	45	44	46 ± 3
0.0160	37	45	32	38 ± 7	50	48	53	50 ± 3
0.0500	39	43	47	43 ± 4	58	50	52	53 ± 4
0.1600	35	40	43	39 ± 4	49	49	40	46 ± 5
0.5000	38	42	38	39 ± 2	48	48	52	49 ± 2
1.6000	37	37	49	41 ± 7	52	54	58	55 ± 3
5.0000	44	40	53	46 ± 7	49	49	52	50 ± 2
Positive Control	171	186	149	**169 ± 19**	236	258	214	**236 ± 22**

Negative Control: DMSO (100 µL/plate); Positive Control: 4-nitroquinoline-N-oxide (1.0 µg/plate) in the absence of S9-mix and 2-aminoanthracene (20 µg/plate) in the presence of S9-mix.Historical negative in the absence of S9-mix: Range 37–58 (mean ± SD = 45 ± 11). Historical negative in the presence of S9-mix: Range 32–70 (mean ± SD = 44 ± 6). Historical positive in the absence of S9-mix: Range 109–195 (mean ± SD = 177 ± 29). Historical positive in the presence of S9-mix: Range 193–263 (mean ± SD = 210 ± 34)

Table 4
6 h Treatments with the indicated chemicals and 28 h incubation in the presence of CytB, in the presence and absence of S9-mix (*statistically increase $p < 0.05$).

Dose	Citotoxicity					Genotoxicity		% Cytostasis	
FBA (mM)	S9-mix	Mononucleated cells	Binucleated cells	Polinucleated cells	% Citotoxicity	Binucleated cells with micronuclei	% of Binucleated cells with micronuclei	Replication index (RI)	CBPI
0	–	9650	2621	350	0	9	0.3	100.0	1.26
0	+	9755	2874	550	1.1	10	0.4	114.6	1.30
0.01	–	9574	2736	370	−0.8	4	0.15	104.2	1.27
0.01	+	9928	2321	540	2.9	2	0.09	101.1	1.27
0.03	–	9836	2968	350	1.9	6	0.2	106.0	1.28
0.03	+	9547	3128	400	−1.1	3	0.1	114.2	1.30
0.1	–	9328	2523	260	−3.3	2	0.08	95.5	1.25
0.1	+	9725	2626	530	0.8	8	0.3	108.8	1.29
0.3	–	9758	2558	330	1.1	3	0.12	96.7	1.25
0.3	+	9826	2380	270	1.8	3	0.13	88.9	1.23
1	–	9521	2723	600	−1.3	5	0.18	116.1	1.31
1	+	9723	2428	500	0.8	4	0.16	103.0	1.27
3	–	10,236	2761	430	6.1	6	0.22	102.5	1.27
3	+	10,257	2923	500	6.3	5	0.17	109.0	1.29
MitC									
0.15 μg/mL	–	12,345	2521	128	27.9	32	1.3*	70.4	1.19
BAP									
3 μg/mL	+	11,074	2210	110	22.3	42	1.9*	68.9	1.18

Historical negative ($n > 20$ experiments): % micronucleated binucleated cells upon treatment with vehicle of 0.30 ± 0.2 and of 0.4 ± 0.3. in the presence of Cyt B and in the absence of Cyt B, respectively.
Historical positive values ($n > 20$) = % micronucleated binucleated cells upon treatment with 1.2 ± 0.3% upon treatment with MitC in the absence of Cyt B and of 1.7 ± 0.3% for BAP in the presence of Cyt B [range 1.5–3].

Table 5
34 h Treatment with FBA at different concentrations of the indicated chemicals in the presence of CytB and in the absence of S9-mix (*statistically increase $p < 0.05$).

Dose	Citotoxicity					Genotoxicity		% Cytostasis	
FBA (mM)	S9-mix	Mononucleated cells	Binucleated cells	Polinucleated cells	% Citotoxicity	Binucleated cells with micronuclei	% of Binucleated cells with micronuclei	Replication index (RI)	CBPI
0	–	9487	2430	295	0	8	0.33	100.0	1.25
0.01	–	9318	2621	302	2.3	7	0.27	106.5	1.26
0.03	–	9532	2432	287	1.7	2	0.08	99.2	1.25
0.1	–	9278	2050	280	3	6	0.29	90.9	1.22
0.3	–	9001	2460	288	2.5	7	0.28	104.5	1.26
1	–	9120	2555	327	3	5	0.20	108.1	1.27
3	–	9870	1860	300	12	7	0.38	82.7	1.20
ColC									
0.1 μg/mL	–	11,428	2727	87	23.3	60	2.2*	77.4	1.20

Historical negative ($n > 20$ experiments): % micronucleated binucleated cells upon treatment with vehicle of in the absence of Cyt B = 0.29 ± 0.20.
Historical positive values ($n > 20$): % micronucleated binucleated cells upon treatment with for ColC in the absence of Cyt B = 2.0 ± 0.4%.

Butyric acid is a nutrient and its safety is well known. Nevertheless, considering that after *in vitro* simulated oro-gastro-intestinal digestion a residual concentration of FBA is expected to be present in the gastrointestinal tract, the evaluation of its possible toxicity was investigated. Thus, to exclude *in vitro* genotoxicity, the bacterial reverse mutation test was applied first to determine possible point mutations induced by FBA. While the Ames Test is a preliminary test, commonly used for the screening of mutagenic substances, the results of this test are very interesting, as point mutations can be responsible for human genetic diseases, and it is known that point mutations in oncogenes and the tumour suppressor genes of somatic cells are involved in human cancer. In the Ames Test, FBA resulted not to be a mutagen. Considering the number of revertants /plate, their means and standard deviations measured for FBA in *S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2 trp UvrA, in the presence (+S9-mix) and absence (−S9-mix) of metabolic activation, FBA does not show a dose-dependent induction of revertants, signs of mutagenicity, in any of the conditions tested. Indeed a) none of the test concentrations exhibited a statistically significant increase in terms of number of revertants per plate compared with the concurrent negative controls; b) no concentration-related increase, nor any other trend, could be identified, c) all results were below the historical range of negative control data, d) all concurrent positive controls gave a statistically significant increases in terms of number of revertants compared with the concurrent negative controls. Providing that all acceptability criteria were fulfilled, we can conclude that FBA is non mutagenic in the Ames Test.

Finally, as far as the *in vitro* mammalian cell Micronucleus Test is concerned, this is another test recommended by the EFSA to study the genotoxicity of novel foods. The results showed that a) none of the test FBA concentrations exhibited a statistically significant increase in terms of % of binucleates containing micronuclei, compared with the concurrent negative controls; b) no concentration-related increase in terms of % of binucleated cells containing micronuclei, nor any trend could be identified, c) all results were below the 95% CI distribution of the historical negative control data, d) all concurrent positive controls gave a statistically significant increase in % of binucleates containing micronuclei compared with the concurrent negative controls. Providing that all acceptability criteria were fulfilled, FBA should be considered unable to induce chromosome breaks in the Micronucleus Test.

5. Conclusions

The results obtained from this study clearly indicate that FBA is a butyric acid releaser, which is able to increase butyrate serum

concentration in a dose and time dependent manner in both male and female mice, with a profile similar to that obtained from butyric acid as such.

Moreover, the results of the *in vitro* toxicological tests can be considered to be a promising starting point for other *in vivo* toxicological studies. In fact, although interesting, the Ames Test presents the limitation that it is carried out on prokaryotic cells, which differ from mammalian cells, and therefore cannot entirely mimic mammalian *in vivo* conditions. In addition, there are many substances found to be positive in this test which exhibit mutagenic activity in other tests, other substances, found negative in this test, do exhibit mutagenic activity in other tests, and finally, other substances again for which the test overestimates mutagenic activity. Thus, to show the safety of FBA, other *in vivo* toxicological investigation must be performed following the tiered approach.

CRediT authorship contribution statement

MD, MS and RR designed the study, drafted and wrote the manuscript. CS, MD, ADF, and ES performed the *in vitro* and analytical experiments. RR performed the *in vivo* study. MS, NB, and ADF performed the toxicological experiments. PC edited the manuscript. All authors revised and gave their final approval for publication.

Conflict of interest statement

The authors declare no conflict of interest.

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