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Title: Fat concentration and high-pressure homogenization affect chlorogenic acid bioaccessibility and α-glucosidase inhibitory capacity of milk-based coffee beverages

Article Type: Full Length Article

Keywords: coffee; milk; fat concentration; high pressure homogenization; in vitro digestion; α-glucosidase inhibition

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Abstract: This study aimed at investigating the effect of coffee formulation and high-pressure homogenization (HPH) on chlorogenic acid bioaccessibility and α-glucosidase inhibition.
Coffee was added with milk (1:1) containing 0.1, 3.6 or 7.1% fat, homogenized at increasing pressure (0-150 MPa) and in vitro digested. Using milk with the highest fat concentration (7.1%) promoted the formation of smaller particles after HPH treatment, as well as upon digestion. Digested samples with the highest fat content also presented lower ζ-potential, suggesting higher stability. Chlorogenic acids (CGAs) bioaccessibility and α-glucosidase inhibition were evaluated upon in vitro digestion. CGAs bioaccessibility increased from nearly 25% to >50% by adding milk and using HPH. These could promote CGAs micellarization, reducing their susceptibility to degradation during digestion. Properly combined milk and HPH also improved α-glucosidase inhibitory effect. No correlation was found between CGAs bioaccessibility and α-glucosidase inhibition, suggesting that other components may govern antidiabetic properties of coffee.

Dear Editor,

I would like to submit the manuscript entitled *"Fat concentration and high-pressure homogenization affect the structural organization, the chlorogenic acid bioaccessibility and the α-glucosidase activity of milk-based coffee beverages"* by Marilisa Alongi, Sonia Calligaris and Monica Anese for consideration for publication in *Journal of Functional Foods*.

This study is part of a Ph.D. research project aimed at evaluating the effect of technological interventions, i.e. formulation and processing, on the bioactivity of some foods. To this regard, a previous paper (Alongi, M., & Anese, M. 2018. Effect of coffee roasting on *in vitro* α-glucosidase activity: inhibition and mechanism of action. *Food Research International, 111*, 480–487) demonstrated that roasting affected the ability of coffee to inhibit α-glucosidase and thus to exert an antidiabetic effect.

The present work further investigated on coffee ability to inhibit α-glucosidase as affected by formulation, i.e. milk addition, and processing, i.e. high-pressure homogenization. Results showed that a proper combination of these factors can positively affect the bioaccessibility of phenolic compound and the antidiabetic ability of coffee, suggesting the potential of technological interventions as a strategic tool to reduce type 2 diabetes risk.

Best regards

Marilisa Alongi

Detailed response to Reviewers

(Reviewer text is normal and Answer text is in *italics* for each item)

Reviewer #1:

In this manuscript, the authors evaluated the bioaccessibility of chlorogenic acids and the alpha-glucosidase activity of milk-based coffee beverages prepared with the same proportion of milk but modifying the fat content and the conditions of high-pressure homogenization and later subjected to an in vitro digestion. It is an interesting study, well designed and I would like to highlight the very good discussion of the results performed by the authors, properly connecting their results with current knowledge on the topic. In my opinion, only some minor modifications should be included in the manuscript:

Abbreviations included in the highlights, the abstracts and the keywords should be defined the first time they are mentioned.
The highlights, the abstract and the keywords were modified to address reviewer comment.

Page 5, lines 73-83. This should be summarized; there is no need to provide all the methodological aspects of the study.
This section was reduced as suggested by the reviewer (lines 81-83).

Some previous study with other beverages, such as cocoa beverage and tea, have provided contradictory results on the role of milk on bioaccessibility and even in vivo bioavailability. I think the authors should (briefly) mention how their results connect with these studies, even if these products do not contain the same classes of phenolic compounds.
The discussion was implemented with further considerations (lines 318-323).

Reviewer #2:

Overall Comments to the authors: The authors planned all experiments with the aim to study the Fat concentration and high-pressure homogenization affect the structural organization, the chlorogenic acid bioaccessibility and the <alpha>-glucosidase activity of milk-based coffee beverages. However, I think this topic of manuscript is more suitable food processing technology rather than functional food field. The results could not truly reflect commercial coffee beverages. I recommend this paper for major revision.

Specific comments:
Line 10: the specific of this higher fat concentration is?
Fat concentration was specified as requested by the reviewer (line 11).

Line 45: Are all functional properties due to the chlorogenic acid? Please explain this relationship.
The sentence was rephrased to clarify this point (lines 50-52).

Line 52: The measurement of bio-accessibility should be given in this concept
Details were added in the text as recommended by the reviewer (lines 58-61).

Line 57: healthy properties should be replaced by healthy benefits.
The suggested change was made (line 65).

Line 71: what is the other compounds formed upon roasting? Phenolic compounds?
Details were added in the text (line 79).

Line 86: Coffee brew was dissolved in deionized water (10 mg/mL), however, I think this concentration is too low. The coffee blends drinking on the market is suitable for the powder to water ratio of 1:10 or 1:20. The content of coffee can influence the performance of high-pressure homogenization.
Coffee brew was prepared to obtain a 20 mg/mL concentration, which allowed preparing a beverage containing 10 mg/mL. The latter is the concentration reported by the producer (Nescafè Gran Aroma, Nestlè, Vevey, Swiss) and agrees with data reported in the literature for instant coffee beverages (Mills et al., 2013; Tagliazucchi et al., 2012). The text was modified to clarify this point (line 87; lines 89-91).
With reference to high-pressure homogenization, it is worth to note that the dry matter coming from coffee was much lower (1-2%) than that coming from milk (around 13%), so that HPH performance were reasonably not affected by coffee concentration.

Line 119: The source of method should be given
The reference was added in the text (line 121).

Line 187: reported should be replaced by shown
The suggested change was made (line 186).

Line 204: what is the latter?
The latter was referred to coffee brew. The text was modified to clarify this point (line 203).

Line 329: the present literature should be given
Literature was added (line 336).

Line 364: These factors also affected the ability of coffee to reduce type 2 diabetes risk by inhibiting <alpha>-glucosidase? Please describe in detail
Details about fat concentration and pressure combinations can be found in lines 347-350. Conclusions were modified accordingly (lines 369-371).

Reviewer #3:

The author aimed to investigate the effect of coffee beverage formulation and homogenization pressure on the bioaccessibility of coffee chlorogenic acids and inhibitory effect against <alpha>-glucosidase activity upon in vitro digestion. A two-variable face-centered central composite design was also used to maximize <alpha>-glucosidase inhibitory capacity of digested coffee beverage. The subject matter is interesting and the motivation of designing coffee beverage to improve the health-promoting performances of coffee is meaningful, but there are some mistakes and questions as detailed in the follows.

Materials and methods:
Line 148-149, 160: Please explain what the digested sample concentration is. I saw you used coffee concentration as x-axis in Figure 2 and Figure 4. But initial coffee concentration in coffee brew and coffee-milk beverage are 10 and 5 mg/mL respectively, then they were diluted 2 times at each digestion stage (8 times dilution in total). So what is the final coffee concentration in coffee brew and coffee-milk beverage group after in vitro digestion and how did you get the concentration gradient as you showed in Figure 2 and 4?
Sample preparation and concentrations were clarified in the text (line 87; lines 89-91; lines 159-160).

Results and discussion:
Line 240-242, 329-332: Considering experiment design, it is not logical to conclude that all of observed inhibition against <alpha>-glucosidase only results from coffee and the milk addition could affect coffee inhibitory effect not directly act as a <alpha>-glucosidase inhibitor after digestion. Please supplement in vitro digestion experiments without any food or with milk only as a negative control of coffee brew group and coffee beverage group respectively to understand the role milk played in inhibiting <alpha>-glucosidase as a part of digested sample.
Alpha-glucosidase inhibition was already tested with controls lacking inhibitors and no inhibitory effect against alpha-glucosidase was found. The text was clarified, and details were added (lines 153-154; lines 232-234; lines 326-328).

Reviewer #4: Manuscript Number: JFF-D-19-00655

Comments:
The paper contains some interesting findings, but the manuscript will require major revisions, including proper formatting and rewriting to bring the paper to a suitable state for publication.
On my opinion, this paper is suitable for publication after some improvements; however, the novelty of the work is poor.

Line 1: In title, I would make it short. Is not clear what it means with "structural organization" of what? An example "Fat concentration and high-pressure homogenization effect on chlorogenic acid bioaccessibility and <alpha>-glucosidase activity of milk-based coffee beverages".
The title was modified as suggested by the reviewer.

Abstract: would be interesting to have a small sentence showing study novelty and reasons for this study. Why applying high-pressure homogenization to a beverage? It is common in industry?
As suggested by the reviewer, the abstract was implemented by elucidating the aim of the study (lines 8-9). Details about the industrial application of HPH were added in the introduction section (lines 40-43).

Introduction: There is a lack of information about high-pressure homogenization. I don't understand why applying this technique to mixture both beverages? The way they are mixtured will have a detrimental effect on particle size and the type of emulsion formed. This represents reality? If is in the title is important for work, explain this better.
Since its development in the early '80s (US patent no. 4,533,254), high pressure homogenization (HPH) has been widely applied in the food industry, especially for the fat globule particle size reduction and thus stabilization of dairy emulsions. Actually, this technology does not present a detrimental effect on emulsion, but on the contrary, it allows the formation of smaller and thus more stable micelles, which were also inferred to protect bioactive compounds from degradation during digestion. Details were added in the introduction section (lines 40-43).

Line 73-74: "Based on this, the aim of the study was to investigate the effect of coffee brew formulation (not containing or containing milk at different fat concentrations)"
(with and without milk at different fat concentrations).
The text was modified as suggested by the reviewer (lines 81-82).

Material and Methods
Section 2.2: Is not clear if authors prepare beverage and immediately they went to gastrointestinal tract. There is a time for stabilization? If so did they monitored if the emulsion is stable or if there is some phase's separation before digestion? What samples were submitted to digestion? Only the mixture of coffee and milk? This is not clear in this section.
Both coffee brew and beverages were digested immediately after preparation and no phase separation was observed during this timeframe. Details were added in the text (line 101).

Section 2.3, line 115: Why authors used 10kDa membrane? The pore size is not very high to retain enzymes? Shouldn't they use 3 kDa instead?
To our knowledge, the bioaccessibility upon in vitro digestion has been evaluated based on different techniques. Some authors apply a simple centrifugation step and recover the supernatant (Quintero-Flórez et al., 2017), some authors apply a filtration using 0.2 μm filters (Cardinali, Linsalata, Lattanzio, & Ferruzzi, 2011), and some other authors apply an ultrafiltration: in this case, the cut-off of membranes varies in the range 3-12 kDa (Tagliazucchi, Helal, Verzelloni, & Conte, 2012; Dinnella, Minichino, D'Andrea, & Monteleone, 2007). It is thus reasonable that the 10 kDa cut-off applied in the present research was able to retain enzymes and guarantee a proper evaluation of phenolic compound bioaccessibility and alpha-glucosidase activity.

Line 143: the undigested sample was the same submitted to digestion? Or they were different samples? Because if they were separated samples how authors guarantee the undigested sample had the same concentration in the sample submitted to digestion?
Samples were prepared in a single batch and split between undigested and digestion. Details about sample preparation were reported in the materials and methods section (lines 97-98).

Results and Discussion
Section 3.1.2: Authors do not verify the appearance of degradation products formed from CGAs degradation? The appearance of new peaks formed, like quinic acid increase?
Line 238: what are the possible reasons for digested coffee being better inhibiting glucosidase than undigested? Maybe small molecules formed during phenolics degradation are interacting with the enzyme. This study would require a LC-MS study to make this relation.
The authors are aware that digestion may induce the formation of other compounds besides chlorogenic acids (Baeza, Sarriá, Bravo, & Mateos, 2018). However, one of the aims of the present research was to understand how technological interventions, such as high-pressure homogenization and milk-based formulation, could affect chlorogenic acid bioaccessibility. As reported in the

introduction section, we focused on this class of compounds as they are considered the major responsible for coffee consumption-related health benefits and they represent the most important class of phenolic compounds in coffee (Iwai et al., 2012; Johnston, Clifford, & Morgan, 2003; Clifford & Knight, 2004).
The authors agree with the reviewer about the possible interaction of smaller molecules formed upon digestion with the enzyme and that investigating more in depth the composition of digested coffee is necessary to elucidate the role of coffee components towards alpha-glucosidase inhibition. Indeed, ongoing research is focusing on the identification of degradation products formed from coffee phenolics upon digestion and on their role in coffee health benefits.

Section 3.2: Why authors do not apply the central composite design to find the best conditions of processing for phenolics preservation
As a relationship between phenolic content and alpha-glucosidase inhibition was not found, the authors decided to study the best conditions able to maximize the aspect more specifically related to coffee health benefits, i.e. the alpha glucosidase inhibitory capacity.

Highlights

- Milk fat and high-pressure homogenization (HPH) affected beverage structure and bioactivity
- Higher fat concentration and pressure induced size decrease and ζ-potential increase
- Milk presence and HPH increased chlorogenic acid bioaccessibility from 25% to >50%
- Milk presence and HPH also improved α-glucosidase inhibitory capacity of coffee

Fat concentration and high-pressure homogenization affect chlorogenic acid bioaccessibility and α-glucosidase inhibitory capacity of milk-based coffee beverages

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Abstract

This study aimed at investigating the effect of coffee formulation and high-pressure homogenization (HPH) on chlorogenic acid bioaccessibility and α-glucosidase inhibition.

Coffee was added with milk (1:1) containing 0.1, 3.6 or 7.1% fat, homogenized at increasing pressure (0-150 MPa) and *in vitro* digested. Using milk with the highest fat concentration (7.1%) promoted the formation of smaller particles after HPH treatment, as well as upon digestion. Digested samples with the highest fat content also presented lower ζ-potential, suggesting higher stability. Chlorogenic acids (CGAs) bioaccessibility and α-glucosidase inhibition were evaluated upon *in vitro* digestion. CGAs bioaccessibility increased from nearly 25% to >50% by adding milk and using HPH. These could promote CGAs micellarization, reducing their susceptibility to degradation during digestion. Properly combined milk and HPH also improved α-glucosidase inhibitory effect. No correlation was found between CGAs bioaccessibility and α-glucosidase inhibition, suggesting that other components may govern antidiabetic properties of coffee.

Keywords:

coffee; milk; fat concentration; high pressure homogenization; *in vitro* digestion; α-glucosidase inhibition

1. Introduction

Recent developments in the food and nutrition disciplines have established that the functionality of bioactive compounds strongly depends not only on food composition but also on its structural organization, as well as on the interaction with co-ingested components (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013). Different physicochemical and biochemical mechanisms might be involved in modifying the functionality of bioactive molecules, especially during digestion (McClements & Xiao, 2014). For instance, the rate of release of bioactive components from the matrix, their inclusion in the micelles, as well as their reaction kinetics with other reactants and co-ingested materials in the gastrointestinal tract can boost or decrease their bioactivity (McClements & Xiao, 2014).

Coffee brews represent a suitable study case to illustrate this behavior. After being discovered in the fifteenth century in Ethiopia, within a century, coffee consumption had rapidly spread in Europe and, later, worldwide (Nehlig, 1999). There are significant differences in the consumption pattern throughout the world, including brew preparation and the addition of ingredients, such as sugar and milk. Coffee-milk based beverages can be regarded as emulsions in which the lipid phase, i.e. milk fat, is dispersed in a water phase containing both coffee constituents (mainly melanoidins, organic acids, and flavors) and milk components (mainly whey proteins and caseins).

Dairy emulsions, such as milk-based beverages, are commonly stabilized by the application of high-pressure homogenization (HPH) (Paquin, 1999). Besides the higher stability due to the formation of smaller micelles, these were also presumed to protect bioactive compounds from degradation during digestion (Otemuyiwa, Williams, & Adewusi, 2017).

It is worthy to note that the consumption of ready-to-drink milk beverages supplemented with coffee has increased markedly. Consumers mainly drink coffee as a stimulant, due to its caffeine content, which can increase alertness, energy, and ability to concentrate. Nonetheless, in the literature several health benefits have been attributed to coffee (Ludwig, Clifford, Lean, Ashiharad,

& Crozier, 2014), including the protection against cardiovascular diseases, as well as antihypertensive, anti-inflammatory, immunoprotective, anti-cancer (Palmioli et al., 2017), anti-aging (Amigoni et al., 2017), neuroprotective (Ciaramelli, Palmioli, & Airoldi, 2019) and antidiabetic (Iwai et al., 2012; Johnston, Clifford, & Morgan, 2003) effects. These have been attributed to chlorogenic acids (quinic esters of hydroxycinnamic acids, CGAs), which are the most important class of coffee polyphenols (12–18% dry weight in green coffee) and can be clustered into three main groups: caffeoylquinic acids (CQAs), with 5-O-caffeoylquinic acid (5-CQA) being the most abundant, feruloylquinic acids (FQAs), and di-caffeoylquinic acids (di-CQAs) (Clifford & Knight, 2004). It is noteworthy that CGAs bioactivity is definitively affected by the digestion process (Baeza, Sarriá, Bravo, & Mateos, 2018) and may be related to their bioaccessibility, i.e. their ability to reach the small intestine upon digestion, as defined by Ferruzzi (2010). The bioaccessibility is commonly assessed trough *in vitro* simulation of the digestion process and is computed as the ratio between the concentration of the compounds found after the intestinal digestion phase and those originally present in undigested food (Ferruzzi, 2010). Coffee digestion was actually simulated in few studies dealing with phenolic compound bioaccessibility and revealed that less than 30% of coffee phenolic compounds was bioaccessible (Podio et al., 2015; López-Froilán, Ramírez-Moreno, Podio, Pérez-Rodríguez, Cámara, Baroni, Wunderlinc, et al., 2016).

Beside these health benefits, chlorogenic acids resulted also able to inhibit α-glucosidase, a hydrolase located on the intestinal cell membrane of the ciliated epithelium, responsible for the release of glucose from oligo- and disaccharides (Chiba, 1997) and thus targeted by several antidiabetic drugs (Akkarachiyasit, Charoenlertkul, Yibchok-Anun, & Adisakwattana, 2010). However, most studies refer to model systems, such as phenolic compounds purified from coffee (Murase et al., 2012; Iwai et al., 2012; Johnston et al., 2003), whereas only a few papers deal with the whole beverage, which includes also other molecules (Moreira, Nunes, Domingues, & Coimbra, 2012). Additionally, most of these studies refer to undigested matrices; whereas to our knowledge no data are available on the ability of coffee to inhibit this enzyme upon the digestion process. To

understand which was the effect of the whole food (i.e. coffee brew) and of processing (i.e. roasting) against type 2 diabetes, in a previous work we investigated the ability of brews obtained from differently roasted coffees towards *α*-glucosidase inhibition (Alongi & Anese, 2018). Results highlighted that the brew capability in inhibiting the enzyme increased with the increase in the roasting degree. Surprisingly, no correlation was found between the ability to inhibit *α*-glucosidase and the chlorogenic acid content, suggesting that other compounds formed upon roasting, such as melanoidins, may play a role in determining the antidiabetic effect of coffee (Iwai et al., 2012; Johnston et al., 2003).

Based on this, the aim of the study was to investigate the effect of coffee brew formulation (with and without milk at different fat concentrations) and HPH on CGAs bioaccessibility and inhibitory effect against *α*-glucosidase activity upon *in vitro* digestion.

2. Materials and methods

2.1. Sample preparation and high-pressure homogenization

Coffee brew was prepared by dissolving instant coffee (Nescafè Gran Aroma, Nestlè, Vevey, Swiss) in deionized water (20 mg/mL). Skimmed milk (0.1% fat, w/w) and cream (35% fat, w/w) (Granarolo S.p.A, Bologna, Italy) were purchased on the local market and properly mixed to obtain milk containing 3.6% and 7.1% (w/w) fat, respectively. Coffee brew (50%, w/w) and milk containing 0.1, 3.6 and 7.1% fat (50%, w/w) were mixed to obtain 10 mg/mL coffee beverages with low, intermediate and high-fat content, respectively. These beverages were homogenized by using a continuous lab-scale high-pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) supplied with two PS type homogenization valves with a flow rate of 10 L/h was used to treat 150 mL of sample. The first valve was the actual homogenization stage and was set at 50, 100 and 150 MPa. The second valve was set at a constant value of 10 MPa. At the exit of the homogenizer, the samples were forced into a heat exchanger set at 4 °C (GEA Niro Soavi, Parma, Italy) to cool

samples to T < 20 °C. Coffee brew and beverages were aliquoted to obtain samples from a same batch to be directly analyzed or to be *in vitro* digested before analyses.

2.2. *In vitro digestion*

In vitro digestion was carried out on coffee brew and coffee beverages according to the protocol proposed by Minekus et al. (2014), immediately after preparation. Briefly, the simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids were prepared and stored at 4 °C. The fluids were preheated to 37 °C just before *in vitro* digestion. The oral phase was started by adding to the sample an α-amylase solution prepared in SSF and providing 75 U/mL activity in the final mixture, $CaCl_2(H_2O)_2$ (0.3 M) to achieve 0.75 mM in the final mixture and SSF. The final ratio of food to SSF was 50:50 (v/v). The sample was maintained at 37 °C under stirring for 2 min. The gastric phase was then started by mixing 5 parts of bolus with 4 parts of SGF, a pepsin solution prepared in SGF and providing 2,000 U/mL activity in the final mixture and $CaCl_2$ to achieve 0.075 mM in the final mixture. The pH was adjusted to 3.0 with HCl (1 M) and water was added to achieve a final ratio of bolus to SGF of 50:50 (v/v). The mix was stirred at 37 °C for up to 2 h. Five parts of chyme were mixed with 4 parts of SIF, a pancreatin solution prepared in SIF and providing 100 U/mL activity in the final mixture, bile salts prepared in SIF and providing 10 mM concentration in the final mixture and $CaCl_2$ to 0.3 mM in the final mixture. The pH was adjusted to 7.0 with NaOH (1 M) and water was added to achieve a final ratio of chyme to SIF of 50:50 (v/v). The mix was stirred at 37 °C for up to 2 h. At the end of the intestinal phase, samples were subjected to ultrafiltration with 10 kDa cut-off (Vivaspin 500, Sartorius, Varedo, Italy) at 13000 *g* for 25 min at 4 °C (Hittich MIKRO 20 Centrifuge, Tuttlingen, Germany). The ultrafiltered sample was considered as the bioaccessible fraction (Tagliazucchi, Helal, Verzelloni, & Conte 2012).

2.3. *Particle physicochemical characterization*

The particle size distribution of digested samples was measured by dynamic laser light scattering (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK), as reported by (Zou et al., 2016).

Samples were diluted 1:1000 (v/v) with deionized water and placed in a cell where the laser light, set at 173 ° angle, was scattered by the particles. Particle size was reported as volume-weighted mean diameter in nm. The ζ-potential was also measured by placing the diluted samples in a capillary cell equipped with two electrodes to assess particle electrophoretic mobility.

2.4. Chlorogenic acid quantification and bioaccessibility

Chromatographic quantification of chlorogenic acids was performed on undigested and digested samples following the method proposed by Mills, Oruna-Concha, Mottram, Gibson, & Spencer (2013). An HPLC pump (LC-10AT VP, Shimadzu Corporation, Kyoto, Japan) equipped with a diode array detector (SPD-10 AT VP, Shimadzu Corporation, Kyoto, Japan) and with an inverse phase apolar C18 column (5 µm, 250 x 4.6 mm, Alltima, Lokeren, Belgium) was used. The injection valve (Rheodyne, Sigma-Aldrich, Milano, Italy) was equipped with a 20 µL plastic loop and samples were injected using a syringe (SGE LC, 100 µL, FN). The elution was carried at a flow rate of 1 mL/min in gradient mode using 5% methanol (Sigma-Aldrich, Milano, Italy) and 95% water containing 0.1% HCl 5 N (solvent A), and 50% acetonitrile (Sigma-Aldrich, Milano, Italy) and 50% water containing 0.1% HCl 5 N (50%) (solvent B) as mobile phase. Gradient was set as follows: solvent A was held at 95% for the first 5 min, decreased to 50% and held at this level up to 40 min; then decreased further to 0% and held up to 59.9 min; finally, 95% solvent A was reached and held up to 60 min. The detection was conducted at 320 nm. Quantification was carried out using external standards. Calibration curves were linear (R^2 > 0.995) in the 2.0 to 200.0 mg/L concentration interval. Peak integration was performed by using Polyview 2000 software (Ver. 5.3, Varian, Texas, USA).

Bioaccessibility was determined as the ratio between the concentration of the compound in the digested sample and that in the undigested sample and results were expressed as a percentage.

2.5. *α*-Glucosidase inhibition

The inhibitory activity of digested coffee brew and beverages against *α*-glucosidase was assessed spectrophotometrically (UV-2501PC, UV-VIS Recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan), as previously described (Alongi & Anese, 2018). Different aliquots of sample were introduced in 1 mL capacity cuvettes in the presence of 30 µL *α*-glucosidase solution (0.04 mg/mL in 0.1 M phosphate buffer, pH=7, corresponding to 1 U/mL), and phosphate buffer (100 mM, pH 7) to the volume of 900 µL, and mixed well. After incubation at 37 °C for 10 min, the reaction was started by adding 100 µL of 5 mM 4-nitrophenyl-*α*-D-glucopyranoside (Sigma-Aldrich, Milano, Italy) solution in 100 mM phosphate buffer (pH 7.0) as substrate. Absorbance was recorded at 405 nm during 10 min after every 30 s. Controls lacking inhibitors (i.e. digestive mixture without sample and digested milk) were run and defined the control activity in each experiment. The *α*-glucosidase inhibition carried out by digested samples was calculated using Equation 1:

$$Inhibitory\ activity\ (\%) = 100 - \left(\frac{k_s}{k_c} \times 100\right) \qquad \text{Equation 1}$$

where k_s and k_c were the kinetic constants in the presence and in the absence of the inhibitor (i.e. digested coffee brew and beverages), respectively. The inhibitory activity (%) against *α*-glucosidase was plotted *vs* the concentration of digested sample (0 to 2.5 mg/mL for coffee brew and 0 to 1.25 mg/mL for coffee beverages), and a logarithmic model was used to fit data so that the half-maximal inhibitory concentrations (IC_{50}), i.e. the concentration of sample required to produce a 50% inhibition against *α*-glucosidase, was calculated.

2.6. *Polynomial equations and statistical analysis*

Modeling was aimed at identifying the combination of fat concentration and pressure able to optimize the inhibitory activity against *α*-glucosidase, by minimizing the IC_{50}. In particular, a 2-factors face-centered central composite design (CCF) was used. The factors considered were fat

concentration and pressure and were set at 0.1, 3.6 and 7.1% (w/w) and at 50, 100 and 150 MPa, respectively. The CCF was completed by a central point (combination of the intermediate values of the two factors). All the factorial points were replicated once, while the central point was replicated 3 times (Table S1). A second order response surface was fitted to the observed data according to Equation 2.

$$y = B_0 + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} B_{ii} x_i^2 + \sum_{j>i\geq 1}^{k} B_{ij} x_i x_j \qquad \text{Equation 2}$$

where B_0 is a constant, B_i, B_{ii}, and B_{ij} are regression coefficients of the model, x_i and x_j are the independent variables in coded values, and k is the number of factors.

Shapiro-Wilk test was used to evaluate normality of the data, while the possible presence of outliers and the homogeneity of variance were evaluated by residual analysis. The goodness of fit was measured with the adjusted determination coefficient (R^2_{adj}). p-Values for the coefficients of the response surface were defined using standard t-test. Contour plot was drawn to illustrate the effect of the considered factors on the IC_{50}.

Results are averages of three measurements and are reported as mean value ± standard deviation. Analysis of variance (ANOVA) was performed by using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance and Tukey test was used to test for differences between means ($p < 0.05$).

3. Results and discussion

3.1. *Effect of* in vitro *digestion on coffee brew*

3.1.1. *Chemical and physical properties*

As shown in Fig. 1, coffee brew presented a multimodal particle size distribution, revealing the presence of two families of compounds, with an average diameter corresponding to 255 ± 10 and 5560 ± 16 nm, respectively. The smaller family could be represented by phenolic compound-protein aggregates, while larger particles could have formed during coffee powder production, upon water evaporation, which is able to induce the irreversible aggregation of coffee components (Le

Bourvellec & Renard, 2012; Gmoser et al., 2017). Upon *in vitro* digestion, a single family of compounds (298 ± 5 nm) was observed. The latter might be formed as a result of the digestive enzyme activity, leading to the degradation of native coffee brew particles and the formation of new ones. In particular, the presence of bile salts, together with amphiphilic molecules deriving from coffee (i.e. fatty acids and phospholipids), could lead to the formation of mixed micelles, containing several digestion products (Salvia-Trujillo et al., 2017).

A significant change ($p < 0.05$) in the ζ-potential was also observed upon *in vitro* digestion. In particular, non-digested coffee presented a value of -17.45 ± 1.34 mV, while the ζ-potential of digested sample was -32.45 ± 1.63 mV. Such a difference can be attributed to the presence of bile salts and other digestion products on mixed micelle surface, leading to higher stability of the digested system, due to particle repulsion (Salvia-Trujillo et al., 2013).

3.1.2. Chlorogenic acids and bioaccessibility

As *in vitro* digestion induced changes in the structural properties of coffee brew, this was also analyzed for chlorogenic acid (CGAs) content, since these compounds represent the most abundant class of polyphenols in coffee (Clifford, 1985; Mills et al., 2013).

As shown in Table 1, two major families of CGAs, namely caffeoylquinic acids, i.e. 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA) and 5-caffeoylquinic (5-CQA), and feruylquinic acids, i.e. 5-feruylquinic (5-FQA) and 4-feruylquinic (4-FQA), were identified and quantified. As expected, 5-CQA was the most abundant compound, and, together with 3-CQA, accounted for more than 70% of the overall CGAs, in agreement with previous data showing that these compounds represent more than half of coffee CGAs (Alongi & Anese, 2018). A significant reduction, up to 80%, in CGAs concentration was observed upon *in vitro* digestion (Table 1). These results agree with data reported by other authors for coffee (Olthof, Hollman, & Katan, 2001; Podio et al., 2015; López-Froilán et al., 2016), as well as for other foods such as tea (Record & Lane, 2001) and fruit juices (Cilla, González-Sarrías, Tomás-Barberán, Espín, & Barberá, 2009). Such a reduction could

be due not only to the activity of digestive enzymes but also to the low stability of phenolic compounds to pH modification during the digestion process. In addition, phenolic compounds can undergo several reactions during *in vitro* digestion, such as polymerization, epimerization, and auto-oxidation, as well as complexation with metal ions present in the digestive mixture (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014). Nonetheless, the phenolic profile of digested coffee resulted analogous to that of the undigested sample, with 5- and 3-CQA still representing the 70% of overall CGAs (Table 1). To better understand which was the ratio of phenolic compounds surviving the digestion process and thus available for uptake by the intestinal mucosa (Ferruzzi, 2010), their bioaccessibility was computed (Table 1). The latter accounted for less than 25% and no significant differences were observed among single CGAs, that presented a bioaccessibility always lower than 30%, suggesting that all CGAs seemed equally susceptible to degradation upon *in vitro* digestion.

3.1.3. α-Glucosidase inhibition

Previous research demonstrated that undigested coffee is able to inhibit *α*-glucosidase (Alongi & Anese, 2018). However, this enzyme is located at intestinal level (Chiba, 1997) and thus it can come in contact only with digested food. As changes in the physical and chemical properties (Fig. 1) as well as in CGAs content (Table 1) were observed upon *in vitro* digestion, the digested coffee brew was tested for its ability to inhibit *α*-glucosidase (Fig. 2). A control represented by the digestive mixture not containing coffee brew was also tested and did not present any inhibitory effect in the whole range of analyzed concentrations (data not shown). On the contrary, the inhibition carried out by digested coffee brew against *α*-glucosidase increased in a concentration-dependent manner (Fig. 2) with an IC_{50} of 0.94 mg_{dw}/mL. The latter was up to 3-fold lower than that reported in the literature for undigested coffee beverages (Alongi & Anese, 2018). In other words, digested coffee would be more efficacious than undigested one in inhibiting *α*-glucosidase, despite the significant decrease in chlorogenic acid concentration observed upon *in vitro* digestion

(Table 1). To this regard, it is noteworthy that the same authors observed an inverse correlation between chlorogenic acid content and α-glucosidase inhibition and suggested that other compounds, such as Maillard reaction products, besides phenolic ones may play a role in inhibiting the enzyme.

3.2. Effect of the addition of milk on coffee properties upon in vitro *digestion*

As coffee is often consumed with the addition of milk and this was demonstrated to affect the bioaccessibility of phenolic compounds (Tagliazucchi et al., 2012), in the second part of this research the effect of milk addition, and relevant fat content, and particle size distribution on coffee inhibitory capacity against α-glucosidase was evaluated. In particular, coffee was added with milk (1:1 w/w) having a different fat concentration (0.1, 3.6 and 7.1%), homogenized with high pressures (50, 100 and 150 MPa) and *in vitro* digested. Table 2 shows the particle size distribution of coffee beverages before and after *in vitro* digestion. Increasing fat concentration to 7.1% produced an overall reduction in the average particle diameter of coffee beverages before *in vitro* digestion. HPH also affected the particle size distribution. When considering the lowest fat concentration (<0.1%), peaks can be mainly related to milk protein aggregates that, upon HPH treatment, were disrupted as evidenced by the change from monomodal to multimodal particle distribution. On the contrary, in beverages containing milk at 3.6 and 7.1% fat, the observed signals can be regarded to both fat globules and proteins aggregates. After HPH treatments the monomodal distribution was maintained with a significant shift to lower particle sizes. In particular, by increasing the pressure, a reduction in average particle diameter from 955 to 396 nm was noted for the 3.6% fat containing beverage and from 190 nm to 106 nm in the case of that with 7.1% fat. As expected, the mechanical forces suffered by the product during HPH process induced the disruption of dispersed particles, giving a reason for emulsions with a different structural organization.

After *in vitro* digestion, three particle families were identified: the first ranging from 30 to 70 nm, the second from 220 to 300 nm and the last around 5500 nm. Particles with an average diameter of

295 nm were also observed in digested coffee brew (Fig. 1) and were attributed to the presence of mixed micelles. The occurrence of smaller and larger particles upon digestion of coffee beverages could instead be attributed to the presence of milk. Several authors (Garcia, Antona, Robert, Lopez, & Armand, 2014; Mercan, Sert, & Akin, 2018; Hayes & Kelly, 2003) observed two major particle families, with average diameter in the order of magnitude of 100 and 10,000 nm, respectively, upon homogenization and *in vitro* digestion of milk. The first family could be represented by fat globules, while the second might include both aggregates of fatty acids, bile salts and undigested fat particles (Singh, Ye, & Horne, 2009; Salvia-Trujillo et al., 2013), as well as digested milk proteins (Koutina, Ioannidi, Melo Nogueira, & Ipsen, 2017). Interestingly, the higher was the fat content, the higher was the volume of smaller particles also after *in vitro* digestion (Table 2). These results suggest that the presence of higher fat concentrations could not only promote the formation of smaller particles upon HPH treatment but also induce the development of smaller micelles during digestion. To this regard, Salvia-Trujillo et al. (2013) observed that increasing the lipid surface area exposed to pancreatic lipase led to a decrease in droplet size obtained upon digestion and to a higher extent of lipid digestion.

To understand if the differences observed in particle size distribution also affected the stability of undigested and digested coffee beverages, the latter were further analyzed for their ζ-potential (Fig. 3). Overall, HPH did not affect the ζ-potential of undigested coffee beverages, while it decreased as fat concentration increased from 0.1 to 3.6% (Fig. 3). A further increase to 7.1% of fat did not modify the ζ-potential. *In vitro* digestion only produced minor changes in the ζ-potential when lower fat concentrations were considered. On the contrary, an outstanding difference was observed in the ζ-potential upon *in vitro* digestion of the coffee beverage containing the highest fat concentration, with a decrease ranging from 2- to 3-fold (Fig. 3c). It is noteworthy that samples presenting the lowest ζ-potential (Fig. 3c), and thus potentially high stability, also presented the smallest particle size distribution upon *in vitro* digestion (Table 2), suggesting that high fat concentration would promote the formation of more stable digesta. To this regard, Otemuyiwa et al.

(2017) reported that the presence of fat could aid the stabilization of the system during digestion by favoring mixed micelles stabilization and solubilization. These authors also reported that the addition of milk, and thus the presence of fat, plays a crucial role in enhancing phenolic availability of tea and cocoa infusions, by helping their micellarization. On the contrary, other authors reported that the presence of milk may impair phenolic compounds bioaccessibility mainly due to the binding with milk proteins (Dupas, Baglieri, Ordonaud, Tomè, & Maillard, 2006; Duarte & Farah, 2011). To get an insight into the effect of the presence of milk with different fat concentration as well as of HPH treatment on chlorogenic acids, the latter were quantified in coffee beverages before and after *in vitro* digestion and their bioaccessibility was computed (Table 3). As shown in Table 3, the bioaccessibility of chlorogenic acids in coffee beverages was strongly affected by the presence of milk. In all cases, the addition of milk increased dramatically the bioaccessibility of CGAs moving from about 20-25% (Table 1) to values higher than 50%, up to more than 100%. These results highlighted the reduced susceptibility of CGAs to degradation upon *in vitro* digestion, as compared to coffee without milk, in which up to 80% of CGAs was lost after digestion (Table 1). To this regard, Duarte & Farah (2011) reported that the effect of milk on CGAs content upon digestion would depend on the milk to coffee ratio, and thus on the fat to coffee ratio, which can result in a positive or negative effect on CGAs bioaccessibility. In our experimental condition, the 1:1 ratio between milk and coffee may considerably improve the health-promoting performances of coffee. Moreover, the bioaccessibility was shown to be affected both by fat content and HPH intensity. Overall, an increase in phenolic bioaccessibility was observed when coffee beverages contained milk with 0.1% fat and were treated at 50 MPa, as well as when 3.6% fat was considered and the beverage was treated at 100 MPa. This pressure, however, when combined with the lowest (i.e. 0.1%) and the highest fat level (i.e. 7.1%) led to a significant decrease in chlorogenic acid content, which accounted for nearly 50% of total CGAs. Different factors could concomitantly affect the CGAs bioaccessibility. As already pointed out, the presence of fat could reduce the susceptibility of CGAs to degradation by promoting their micellarization (Otemuyiwa et al., 2017).

In addition, phenolic compounds may complex with milk proteins and thus result less prone to breakdown during digestion (Dupas et al., 2006; Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014). Finally, the system particle size could modulate the digestion kinetics and thus CGAs bioaccessibility (Salvia-Trujillo et al., 2013).

Altough contradictory effects were reported in the literature regarding the simultaneous consumption of milk with phenolic compounds on their bioaccessibility (Duarte & Farah, 2011; Tagliazucchi et al., 2012), this controversy was explained by Del Rio, Borges, & Crozier (2010) considering the different concentration of phenolic compounds. According to these authors, milk could interfere with the absorption in the case of low phenolic concentration, but it could have an opposite effect if the phenolic concentration is high enough, as in the case of coffee brew (Del Rio et al., 2010).

Since the addition of milk to coffee brew induced changes in the physical and chemical properties upon *in vitro* digestion, digested coffee beverages, as well as digested milk samples, were further analyzed for the inhibition against α-glucosidase (Fig. 4). Digested milk did not inhibit the enzyme in the whole range of analyzed concentrations (data not shown), while digested coffee beverages inhibited α-glucosidase in a concentration-dependent manner (Fig. 4). The highest efficacy was observed for the beverage containing milk with 7.1% fat and treated at 100 MPa. Overall, the use of higher pressures (i.e. 100 and 150 MPa) improved the α-glucosidase inhibitory capacity of all beverages. The fat content also affected the ability to inhibit the enzyme. It can be noticed that when 3.6% fat was used, all coffee beverages showed a lower inhibitory capacity against α-glucosidase as compared to coffee brew (Fig. 2). On the contrary, lower (0.1%) or higher (7.1%) fat levels improved the ability of coffee beverages to inhibit the enzyme. To our knowledge, despite some information is present in the literature regarding the effect of milk addition on the bioaccessibility of coffee phenolic compounds (Tagliazucchi et al., 2012), for the first time, these data demonstrated that milk addition may affect coffee ability to inhibit α-glucosidase.

3.3. Identification of the best fat-pressure combination able to minimize IC_{50}

To define the best performing process conditions to obtain a coffee beverage with improved α-glucosidase inhibitory capacity, a 2-factors face-centered central composite design (CCF) was used. To this aim, fat concentration and pressure were considered as independent variables and their effect on IC_{50} was studied (Table S1). The regression coefficients and the relative analysis of variance of the polynomial models for the dependent variable, namely IC_{50}, were computed (Table S2). Finally, to evaluate the effect of the independent variables on the dependent one and to predict the optimum values of each variable for minimum IC_{50} to be achieved, a contour-plot was generated (Fig. 5). As reported in Fig. 5, the IC_{50} could be minimized by different combinations of fat concentration and pressure. In particular, when a low lipid concentration was used (<0.5%), higher pressures (>125 MPa) would be required to minimize the IC_{50}. On the other hand, when a higher lipid concentration was used (around 7%), the IC_{50} could be minimized by applying intermediate pressures (between 80 and 110 MPa). When these conditions were applied, an IC_{50} lower than 0.5 mg_{dw}/mL was obtained. This means that a proper combination of fat concentration and pressure could almost double the ability of coffee to inhibit α-glucosidase since the IC_{50} of coffee brew without milk was 0.94 mg_{dw}/mL.

However, it is noteworthy that the combinations able to minimize the IC_{50} led to a significant decrease in CGAs bioaccessibility (Table 3). It can be thus inferred that the inhibitory effect against α-glucosidase might not rely on CGAs content, despite these were reported to present antidiabetic effects through α-glucosidase inhibition (Ishikawa et al., 2007). As already reported in previous work relevant to undigested coffee (Alongi & Anese, 2018), an inverse relationship was found between the phenolic content and the efficacy in inhibiting α-glucosidase. The latter could be carried out by other compounds formed upon processing, among which are melanoidins that can incorporate phenolic compounds (Perrone, Farah, & Donangelo, 2012). Moreover, it can be inferred that even in the presence of a higher bioaccessibility, complexation phenomena occurring between

phenolic compounds and milk proteins upon high-pressure homogenization (Paquin, 1999) and digestion (Hasni *et al.*, 2011; Ali et al., 2012) might result in a reduced bioactivity (Lorenz *et al.* 2007, Serafini *et al.*, 2009).

4. Conclusions

The results acquired in the present study pointed out that both formulation (i.e. the addition of milk with different fat content) and processing (i.e. homogenization pressure) play a key role in determining the bioaccessibility of coffee CGAs. A proper combination of milk and pressure also increased the potential of coffee to reduce type 2 diabetes risk by improving its ability to inhibit α-glucosidase, through IC_{50} minimization. However, this bioactivity was not related to CGAs bioaccessibility, and could thus rely on the presence of other compounds, as well as on more complex interactions occurring upon processing and digestion.

It can be suggested that designing foods aimed at delivering defined functionalities should not only focus on increasing the bioaccessibility of single bioactive compounds but should aim at maximizing the desired bioactivity. To this purpose, the food matrix effect, including the presence of other constituents and their interactions, as well their fate upon digestion must be considered, to properly modulate technological interventions.

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Conflict of interest

All authors declared no conflict of interest.

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Captions for figures

Fig. 1. Particle size distribution of coffee brew, before and after *in vitro* digestion.

Fig. 2. α-Glucosidase inhibitory activity of digested coffee brew as a function of coffee concentration. Data fitting: line, estimates; symbols, experimental data.

Fig. 3. ζ-Potential of coffee beverages containing 0.1% (a), 3.6% (b) and 7.1% (c) of fat and homogenized with different pressure before and after *in vitro* digestion. Lowercase letters (a-b) indicate significant differences before and after *in vitro* digestion, uppercase letters (A-C) indicate significant differences among different pressures within the same sample.

Fig. 4. α-Glucosidase inhibitory activity of digested coffee beverages containing 0.1% (a), 3.6% (b) and 7.1% (c) of fat and homogenized with different pressure as a function of coffee concentration.

Fig. 5. Fitted contour plot of IC_{50} as a function of fat concentration in coffee beverages and homogenization pressure.

Table 1

Concentration before (Undigested) and after (Digested) *in vitro* digestion of coffee brew, and bioaccessibility of 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA), 5-caffeoylquinic (5-CQA), 5-feruylquinic (5-FQA), 4-feruylquinic (4-FQA) and total chlorogenic (Total CGAs) acids.

CGA	Undigested (mg/g_{dw})	Digested (mg/g_{dw})	Bioaccessibility (%)
3 CQA	4.24 ± 0.12 [a]	1.22 ± 0.33 [b]	28.85 ± 7.78 [A]
4 CQA	1.57 ± 0.06 [a]	0.39 ± 0.08 [b]	25.09 ± 5.27 [A]
5 CQA	9.99 ± 0.10 [a]	2.14 ± 0.19 [b]	21.40 ± 1.86 [A]
5 FQA	1.41 ± 0.04 [a]	0.39 ± 0.08 [b]	27.72 ± 6.01 [A]
4 FQA	2.74 ± 0.02 [a]	0.54 ± 0.16 [b]	19.53 ± 5.95 [A]
Total CGAs	19.95 ± 0.30 [a]	4.68 ± 0.77 [b]	23.46 ± 3.87 [A]

Lowercase letters (a-b) indicate significant differences of concentration ($p > 0.05$) between undigested and digested samples, uppercase letters (A) indicate significant differences of bioaccessibility among CGAs.

Table 2

Particle size distribution of coffee beverages containing 0.1%, 3.6%, and 7.1% fat, before and after *in vitro* digestion.

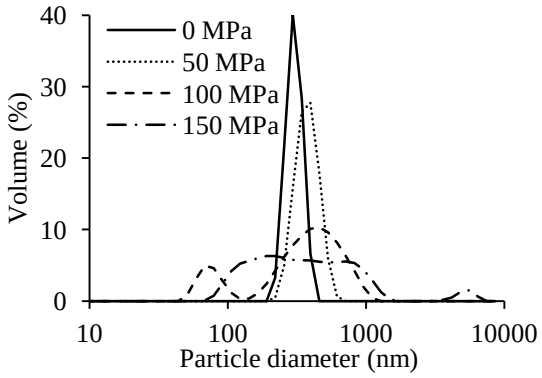
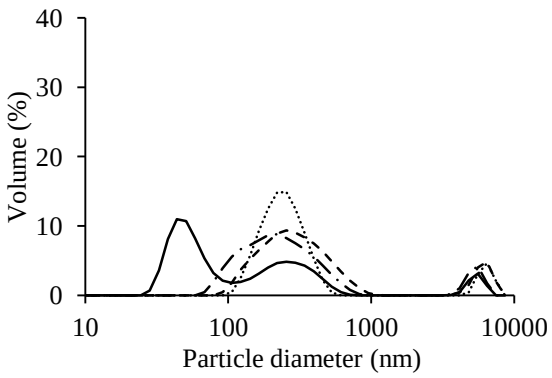
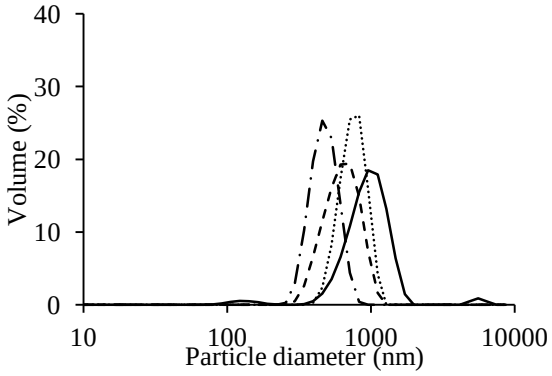
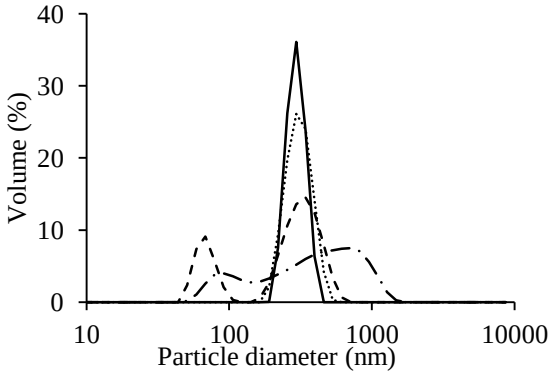
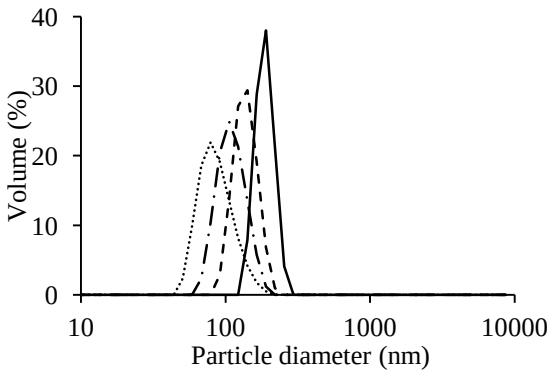
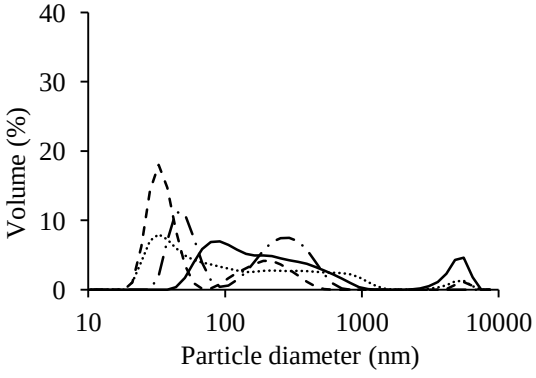
Fat content (%)	Before *in vitro* digestion	After *in vitro* digestion
0.1		
3.6		
7.1		

Table 3

Bioaccessibility (%) of 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA), 5-caffeoylquinic (5-CQA), 5-feruylquinic (5-FQA) and 4-feruylquinic (4-FQA) acids, and total chlorogenic acids (Total CGAs) in coffee beverages with different fat concentration and homogenized with different pressure.

	0.1% fat				3.6% fat				7.1% fat			
CGA	0 MPa	50 MPa	100 MPa	150 MPa	0 MPa	50 MPa	100 MPa	150 MPa	0 MPa	50 MPa	100 MPa	150 MPa
3 CQA	92±1^{ac}	118 ±8^{ab}	57 ±5^{c}	80 ±4^{ac}	98±6^{ac}	95 ±7^{ac}	127 ±25^{a}	137±16^{a}	97±5^{ac}	101±2^{ac}	69±2^{bc}	91±4^{ac}
4 CQA	95 ±22^{ab}	126±4^{a}	59±7^{b}	80±3^{ab}	100±6^{ab}	95 ±19^{ab}	118±25^{ab}	112±1^{ab}	88±2^{ab}	91±2^{ab}	65±1^{b}	86±2^{ab}
5 CQA	90±14^{ab}	104±4^{a}	45±5^{b}	64±1^{ab}	73±5^{ab}	72±18^{ab}	90±19^{a}	76±12^{ab}	72±3^{ab}	69±1^{ab}	47±1^{b}	68±3^{ab}
5 FQA	96±9^{bc}	155±1^{a}	61±5^{c}	73±11^{bc}	84±5^{bc}	83±15^{bc}	104 ±18^{b}	83±13^{bc}	92±17^{bc}	103±2^{bc}	76±2^{bc}	102 ±3^{bc}
4 FQA	105±19^{ac}	105±5^{ac}	59±15^{bcd}	61±9^{bcd}	80±4^{ad}	82±11^{ad}	119±18^{a}	109±29^{ab}	50±3^{d}	52±2^{cd}	38±1^{d}	53±1^{cd}
Total CGAs	93±7^{ab}	112±1^{a}	51±2^{b}	69±1^{ab}	81±5^{ab}	81±18^{ab}	102±19^{a}	93±1^{ab}	77±4^{ab}	78±1^{ab}	54±2^{b}	74±3^{ab}

Different letters (a-d) indicate significant differences.

Table S1

Combination of fat concentration and pressure of different runs and experimental results (half maximal inhibitory concentration, IC_{50}) of a two-factors face-centered central composite design.

Run	Fat concentration (%)	Pressure (MPa)	IC_{50} (mg/mL)
1	0.1	50	2.76 ± 0.56
2	0.1	150	0.70 ± 0.03
3	7.1	50	0.92 ± 0.06
4	7.1	150	1.23 ± 0.13
5	0.1	100	0.92 ± 0.11
6	7.1	100	0.50 ± 0.02
7	3.6	50	2.05 ± 0.55
8	3.6	150	1.22 ± 0.14
9	3.6	100	1.18 ± 0.18
10	3.6	100	1.12 ± 0.13
11	3.6	100	0.81 ± 0.05

Table S2

Regression coefficients of the models for IC_{50} of digested coffee beverages.

Variable	IC_{50}
Intercept	1.014 ***
Fat concentration	-0.581 **
Fat concentration^2	-0.55 *
Pressure	-0.860 ***
Pressure^2	1.299 ***
Fat concentration × Pressure	1.185 ***
R^2_{adj}	0.950

(*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$

Figure 1
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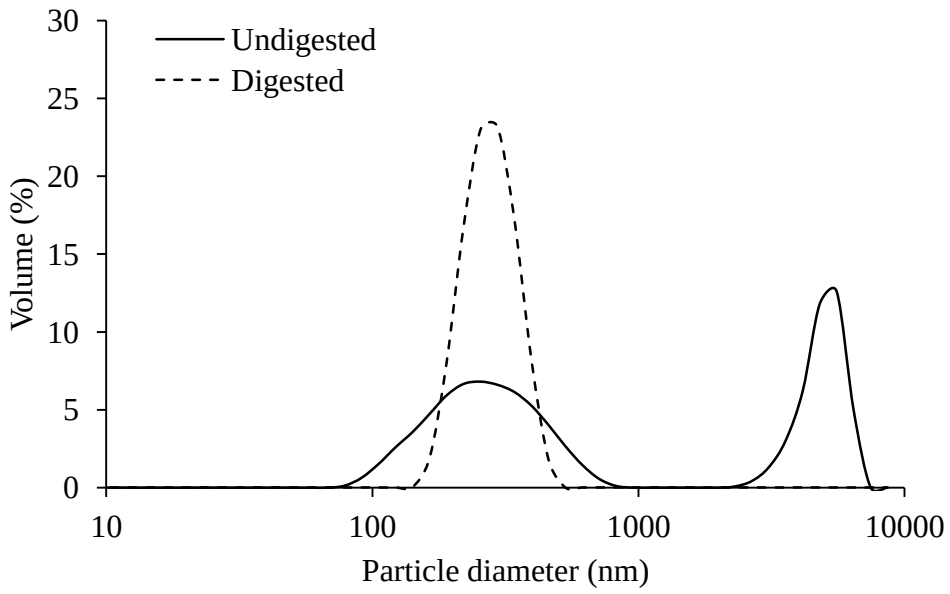

Fig. 1.

Figure 2
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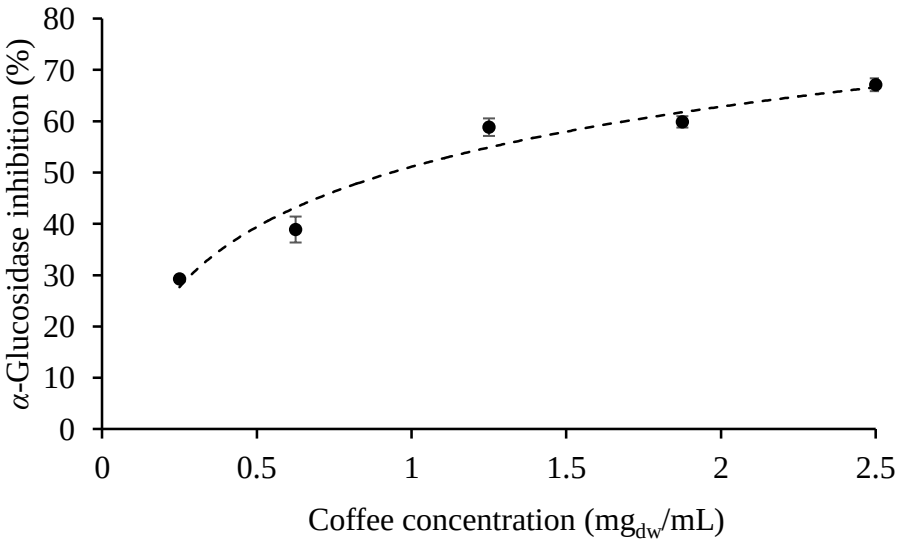

Fig. 2.

Figure 3
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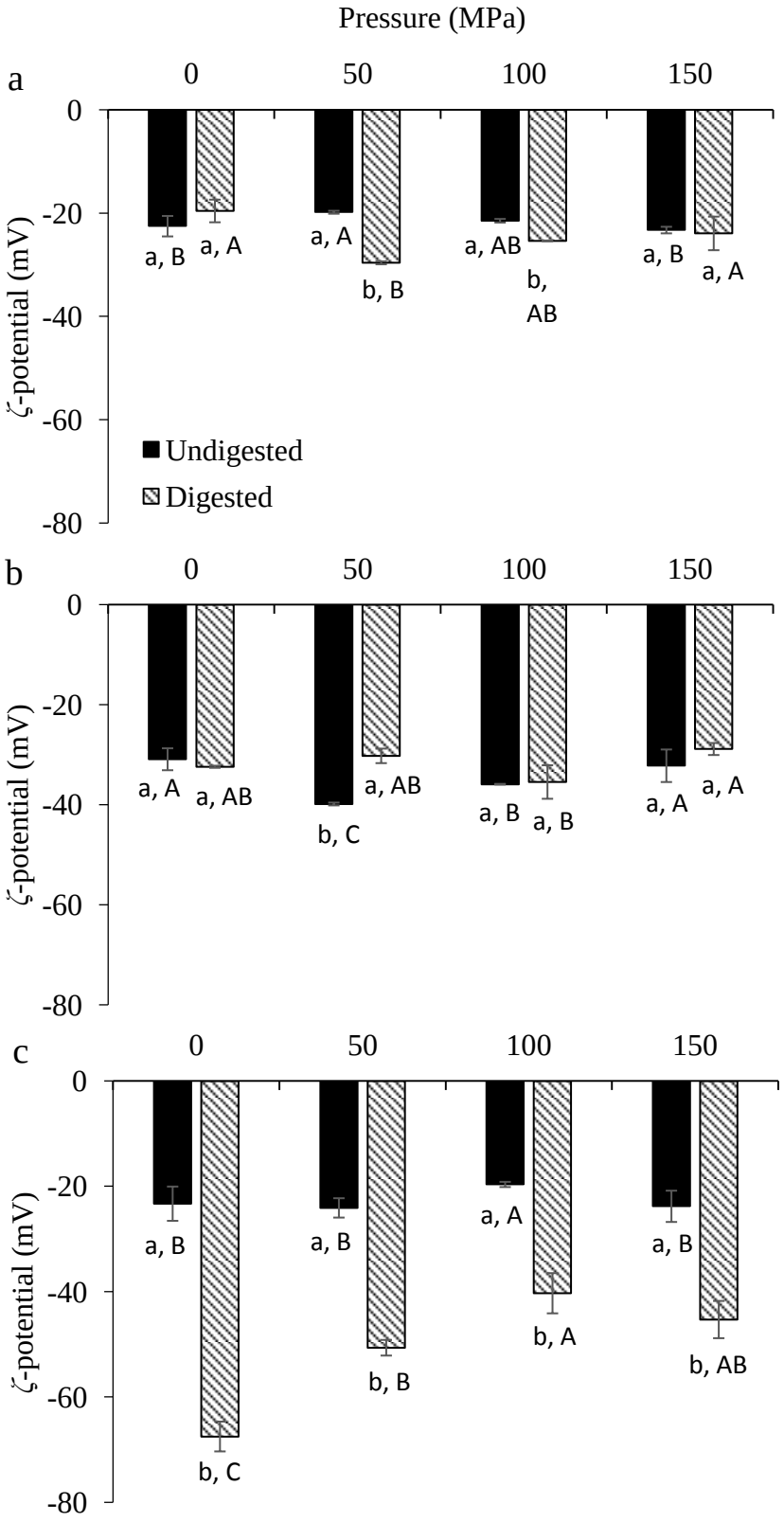

Fig. 3.

Figure 4
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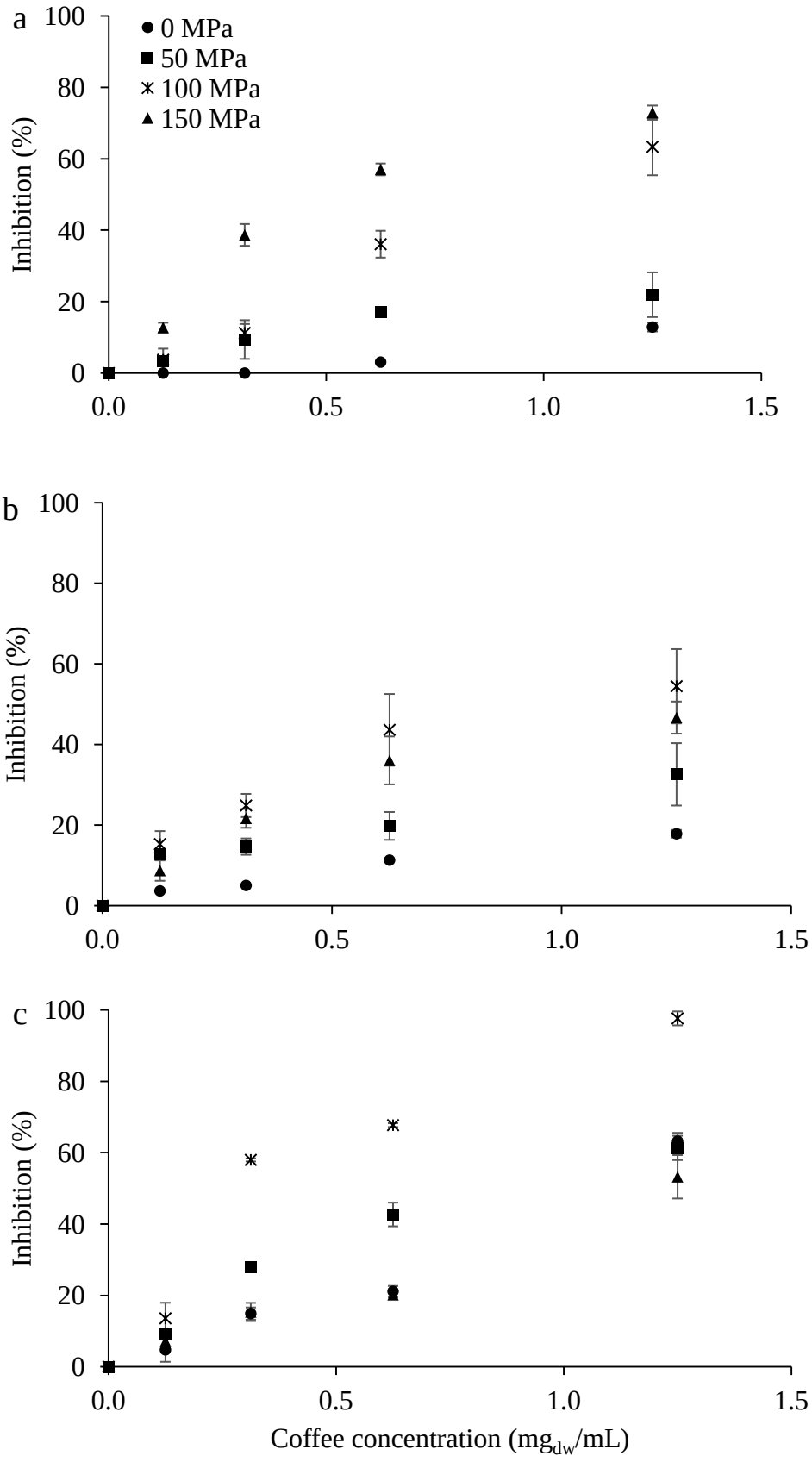

Fig. 4.

Figure 5
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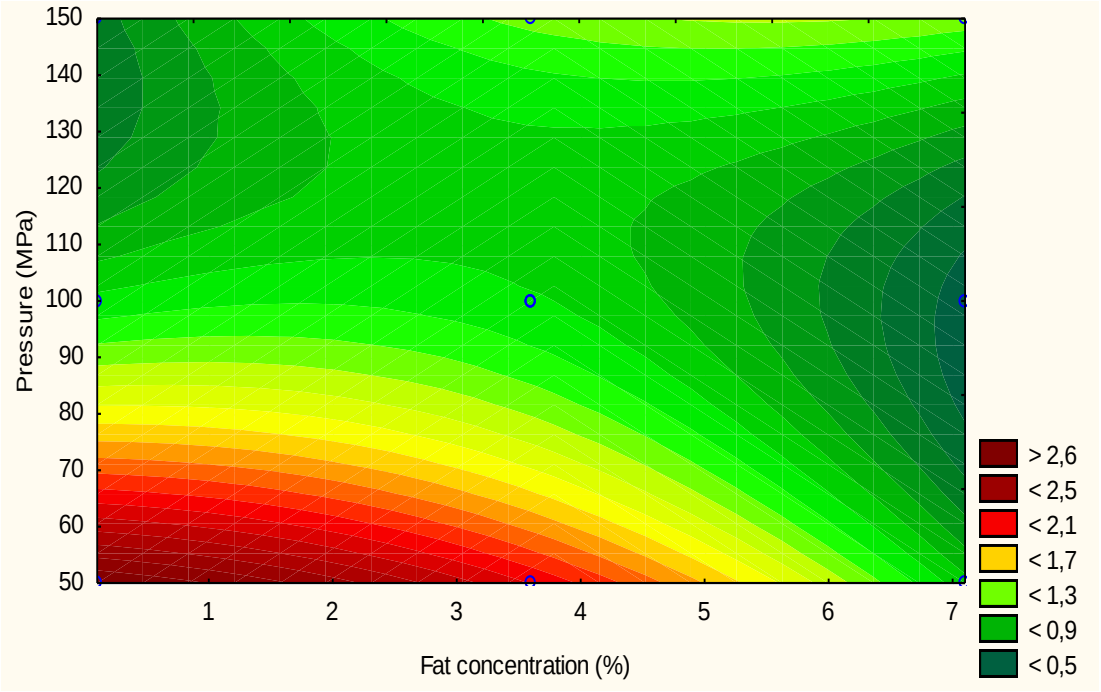
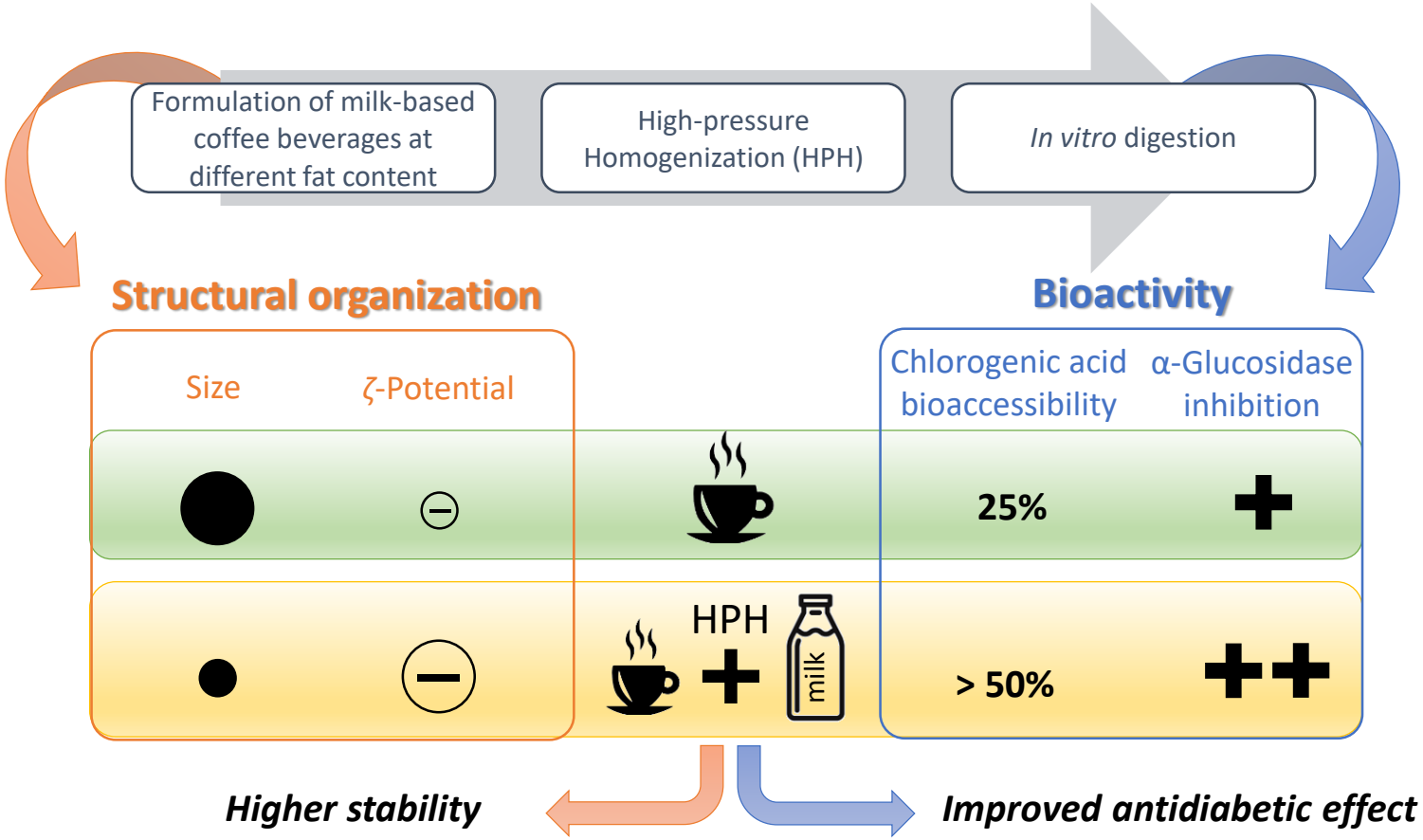


Fig. 5.

*Graphical Abstract



*Conflict of Interest

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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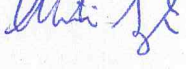
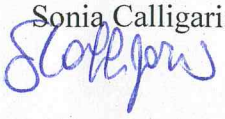
Udine, 13th March 2019

Signed by all authors as follows:

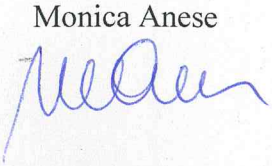
Marilisa Alongi



Sonia Calligaris



Monica Anese



*Ethics Statement

ETHICS STATEMENT

We wish to declare that the present research did not include any human subjects and animal experiments.

Udine, 13th March 2019

Signed by all authors as follows:

Marilisa Alongi

Sonia Calligaris

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