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Phenolic profile and fermentation patterns of different commercial gluten-free pasta during *in vitro* large intestine fermentation

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ABSTRACT

The fate of phenolic compounds, along with short-chain fatty acids (SCFAs) production kinetics, was evaluated on six different commercial gluten-free (GF) pasta samples varying in ingredient compositions, focussing on the *in vitro* faecal fermentation after the gastrointestinal digestion. A general reduction of both total phenolics and reducing power was observed in all samples, together with a substantial change in phenolic profile over 24 h of faecal fermentation, with differences among GF pasta samples. Flavonoids, hydroxycinnamics and lignans degraded over time, with a concurrent increase in low-molecular-weight phenolic acids (hydroxybenzoic acids), alkylphenols, hydroxybenzoketones and tyrosols. Interestingly, discriminant analysis also identified several alkyl derivatives of resorcinol as markers of the changes in phenolic profile during *in vitro* fermentation. Furthermore, degradation pathways of phenolics by intestinal microbiota have been proposed. Considering the total SCFAs and butyrate production during the *in vitro* fermentation, different fermentation kinetics were observed among GF pasta post-hydrolysis residues.

1. Introduction

In the last few years, extensive research has been conducted to investigate the possible health implications of newly developed cereal-based gluten-free (GF) products based on the use of novel ingredients, including, but not limited to, fruit-based extracts, green tea and spice powders in combination with alternative GF flours from cereals, pseudocereals and legumes (Pellegrini & Agostoni, 2015). In particular, GF pasta has been increasingly enriched with different ingredients to provide functional dietary compounds, such as minerals, antioxidant, polyphenols and fibre sources (Marti & Pagani, 2013).

Among functional compounds, antioxidants are very appreciated dietary factors not only in the prevention of several chronic diseases (Macagnan, da Silva, & Hecktheuer, 2016) but also in the enhancement of organoleptic properties of foods (*i.e.*, bitterness, colour and flavour). For instance, epidemiological studies suggested that the regular consumption of dietary polyphenols can be implied in the protection against the development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Zhang & Tsao, 2016). Most reported dietary antioxidants are totally or partially absorbed in the small intestine (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005), and a considerable amount of these molecules are able to pass through the small intestine almost unchanged thanks to the food matrices' carrier-type action, thus reaching the large intestine (Macagnan et al., 2016; Vitaglione, Napolitano, & Fogliano, 2008). Upon reaching the large intestine, microbial enzymes may release antioxidant compounds from complex macromolecules, thus permitting their absorption in their original chemical forms or after further microbial metabolism, which may be responsible for the beneficial health effects of antioxidant-rich food consumption (Costabile, Klinder, Fava, & Napolitano, 2008; Pérez-Jiménez, Serrano, Tabernero, & Arranz, 2009). In addition to free antioxidant compounds from the food matrix, the enzyme activity of the microbial population in the large intestine can enable the breakdown of undigested food residues during anaerobic fermentation, which results in short chain fatty acids (SCFAs) production, mainly acetate, propionate and butyrate (Duda-Chodak, Tarko, Satora, & Sroka, 2015). In particular, butyrate has received considerable attention in the last few years mainly because of the connection between high levels of butyrate in the large intestine and the reduction of several risk factors involved in the development of gut inflammation (Brouns, Kettlizt, & Arrigoni, 2002; Rios-Covián et al., 2016).

Several authors have focussed on the potential application of *in vitro* models in an attempt to re-create *in vivo* conditions (Hein, Rose, Van't Slot, Friedrich, & Humpt, 2008; Juániz et al., 2016). In particular, Mosele, Macià, Romero, Motilva, and Rubió (2015), using *in vitro* gastrointestinal digestion and colonic fermentation models, studied the stability and catabolism of phenolic compounds in pomegranate products,

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whereas Chen et al. (2016), using a similar approach, evaluated the importance of fruit seeds in the prevention of oxidative stress diseases. However, especially for GF pasta formulated with novel ingredients, little is known about the identification and stability of phenolic compounds, as well as the production of SCFAs, following large intestine fermentation. This may be a concern since indications suggest that a GF diet in itself may change the composition, immune function and activity of the gut microbiota (De Palma, Nadal, Collado, & Sanz, 2009).

Therefore, the aim of this study was to apply an *in vitro* faecal fermentation model to investigate the fate of phenolic compounds, along with SCFAs fermentation kinetics, following the consumption of commercially available GF pasta with different ingredient compositions.

2. Materialsandmethods

2.1. Gluten-free pasta and preparations

A set of six commercially available GF pasta samples (respectively, black rice, chickpea, red lentil, sorghum, amaranth and quinoa enriched) was used. Samples were gratefully donated by Gluten Free House (Parma, Italy). For each sample, the ingredients, along with the chemical compositions in accordance with the label indications, are presented in Table 1. In addition, the insoluble and soluble total dietary fibre contents were enzymatically quantified (Megazyme assay kit K-INTDF 02/15) following manufacturer's procedure, and included in Table 1. All samples were analysed 'as eaten' and were freshly prepared prior to the experiment. For each sample, the optimal cooking time was chosen according to manufacturer indications. The GF pasta samples (2 g) were cooked to optimum in 60 ml of boiling water, drained for 1 min, cut into small pieces in a mortar to simulate mastication and then analysed.

2.2. Simulated in vitro gastrointestinal digestion model

The adopted procedure included two distinct phases: a pre-incubation with digestive enzymes followed by an *in vitro* large intestine fermentation process. For the pre-incubation step, cooked to optimum, GF pasta samples underwent an *in vitro* hydrolysis simulating gastric and pancreatic phases as detailed by Giuberti, Gallo, Cerioli, Fortunati, and Masoero (2015). Afterwards, each GF pasta residue was carefully collected by filtration using a nylon cloth (42 μm) and dried overnight at 45 °C. According to the intrinsic degradability of each GF pasta, the pre-incubation step was repeated 6 to 18 times to obtain enough hydrolysed residues for the subsequent *in vitro* large intestine fermentation step. A standard sample of Gelose 80 maize starch (Penford Food Ingredients Co., Colorado, USA) was incubated in each run as an internal standard control check. Residues from different incubations of the same GF pasta samples were pooled.

The large intestine fermentation of the resulting pooled post-hydrolysis GF pasta residues was conducted according to the *in vitro* approach detailed by Giuberti, Gallo, Moschini, and Masoero (2013). Fresh faeces were collected from the rectum of four growing pigs (35 to 46 kg body weight; fed with a standard commercial diet devoid of antibiotics) and placed in airtight plastic syringes and in a water bath at 39 °C. The faecal inoculum, obtained by pooling equal amounts (by wet weight) of fresh faeces from each animal, was mixed with 200 ml of 39 °C CO_2-saturated buffer solution (Menke & Steingass, 1988) using a Stomacher Lab Blender 400 (Seward Medical, Norfolk, UK) and filtered through a 250 μm mesh screen. Thereafter, an additional volume of buffer solution was added to reach the desired dilution of faeces in the buffer (0.05 g faeces/ml buffer) (Jha, Bindelle, Rossnagel, Van Kessel, & Leterme, 2011). Then, 400 mg each dry pooled GF pasta residue was weighted in triplicate into 125 ml glass serum bottle (Z114041, Sigma-Aldrich, Milan, Italy) and filled with 60 ml of the buffer solution containing the faecal inoculum. Bottles were sealed with a rubber stopper and simultaneously placed in a 39 °C shaking water bath (50 rpm). Three bottles without substrate were used as blanks. All manipulations and sample handlings were done under continuous CO_2 flushing. Three incubation runs were carried out in three different days, and bottles within runs were considered repetitions, whereas bottles between runs were considered replicates. Aliquots (3.0 ml) were aseptically removed from each bottle using a 5 ml plastic injection syringe equipped with a catheter needle at 0, 4, 8, 24, 48 and 58 h after incubation and immediately stored at −4 °C for further analysis.

Table 1
Ingredients, along with the label indications on chemical composition (g), of six different gluten-free (GF) pasta samples are reported. Nutritional values are expressed per 100 g of raw product. Superscript numbers refer to the list of ingredients.

GF pasta	Carbohydrates	Proteins	Lipids	Fibre	SDF	IDF
Black rice[1]	74.2	6.8	1.8	2.2	0.8	1.4
Chickpea[2]	72.5	10.7	3.3	5.5	1.6	3.9
Red lentil[3]	71.7	10.8	2.5	5.6	1.4	4.2
Sorghum[4]	72.3	8.4	2.1	2.1	0.5	1.6
Amaranth[5]	74.3	8.4	3.1	4.1	1.7	2.4
Quinoa[6]	76.5	7.4	2.8	7.1	2.5	4.6

1 = black rice flour (50%), white rice flour, corn starch, green tea (0.3%), water. 2 = brown rice flour, water, chickpeas (20%), cumin powder (0.2%). 3 = brown rice flour, water, red lentils flour (20%), turmeric powder (0.3%). 4 = corn flour, white sorghum flour (20%), water. 5 = brown rice flour, amaranth flour (20%), corn starch, water. 6 = brown rice flour, quinoa flour (20%), water. SDF = soluble dietary fibre; IDF = insoluble dietary fibre.

2.3. Bacterial profile of faecal inoculum

DNA extraction of the dominant bacterial communities in fresh faeces was performed as previously described (Miragoli et al., 2016). Afterwards, copy numbers of the 16S rRNA gene from *Bacteroides/Prevotella*, *Clostridium coccoides*, *Bifidobacterium*, *Lactobacillus*, Enterococcus, Enterobacteriaceae, and *Ruminococcus* groups were determined using previously reported primers (Bruzzese et al., 2014), according to a real-time polymerase chain reaction (RT-PCR).

2.4. Extraction of phenolics at different times of in vitro fermentation

Starting from the solution collected from the *in vitro* large intestine fermentation, aliquots of 1.5 ml were taken at 0, 8 and 24 h and extracted in the same volume of 3% formic acid in 70% methanol (LCMS grade, Sigma-Aldrich, Milan, Italy) solution. Each aliquot was stored overnight at −18 °C, centrifuged at 10,000 × *g* for 10 min at 4 °C and then filtered in amber vials using 0.22 μm cellulose syringe filters.

2.5. Evaluation of the phenolic profile

The total phenolic contents were determined colorimetrically according to the Folin-Ciocalteu assay, as previously reported (Lucini, Kane, Pellizzoni, Ferrari & Trevisi, 2016). Briefly, aliquots (33.3 μl) of each extract were mixed with 66.6 μl of Folin reagent (Sigma-Aldrich, diluted fivefold) and 100 μl (75 g l^{-1}) sodium carbonate. Absorbance was recorded at 765 nm after 40 min at 20 °C using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT). A calibration curve was prepared using gallic acid in ethanol and the results consequently expressed as gallic acid equivalents (GAE). Subsequently, phenolic compounds profiling was carried out through high-resolution mass spectrometry, using a hybrid quadrupole-time-of-flight instrument coupled with a UHPLC chromatographic system (UHPLC/QTOF-MS). A 1290 liquid chromatograph coupled with a G6550 mass spectrometer detector *via* a Dual Electrospray Jet Stream ionisation system (all from Agilent technologies, Santa Clara, CA, USA) was used. The mass spectrometer worked in positive scan mode to acquire the range of 50–1000 *m/z*. Chromatographic separation was achieved using a Knauer BlueOrchid C18 column (100 × 2 mm i.d., 1.8 μm) and a mixture of water (proteomic grade, VWR, Milan, Italy) and methanol (LCMS grade, VWR, Milan, Italy) as mobile phase. Formic