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Abstract

Experimental gluten-free (GF) rice cookies were formulated with 100% rice flour (CTR) or by substituting 50 % of rice flour with native waxy rice starch (WRS) or with three different resistant starch (RS) ingredients obtained from debranched, annealed or acid and heat-moisture treated WRS (RSa, RSb and RSc, respectively). Chemical composition, in vitro starch digestibility and physical and textural characteristics were carried out. Among cookies, RSa-cookies had the highest total dietary fibre content, the lowest rapidly digestible starch and the highest RS contents. All the three RS preparations have proved effective in increasing the proportion that tested as RS with respect to native WRS. However, the estimated RS loss for each applied RS ingredients caused by the baking process followed the order of RSa < RSc < RSb. Last, the lowest vitro glycaemic index value was measured for RSa-cookies. Among cookies, differences in colour and hardness were reported. The partial replacement of commercial rice flour with RSa could contribute to formulate GF cookies with higher dietary fibre content and likely slowly digestible starch properties more than equivalent amounts of RSb and RSc.

Keywords Gluten-free; Resistant starch; Predicted glycemic index; Cookie.

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AUTHOR RESPONSE:

The study evaluated the potential nutritional effect of three RS ingredients obtained from WRS in GF cookies. Besides, physical and textural characteristics of cookies were analyzed.

the work is very interesting, the experimental design is appropriate and the results were deeply analyzed and discussed.

AUTHORS (AU): thank you.

However the resistant starches used in GF formulation were not enough characterized. The authors described the procedure and only measured the RS content obtained with each treatment even though they cited previous work with other information about these WRRS. Some additional information will be needed to understand the fundamental changes that led to the nutritional differences.

I think that the manuscript could be improved by adding more information like thermal and viscosity behaviour of WRRS (measured by DSC and RVA) and also water binding capacity. These starch properties would explain the differences in HI, GI, k when cookies (made with RS a b and c) were digested.

AU: according to suggestion, all the analyses were inserted in the revised version of the manuscript (from lines 139 to 154). A new table (table 1) and a new figure (figure 1) are now present containing parameters of interest. Accordingly, results are discussed in lines 254-298.

Gluten free rice cookies with resistant starch ingredients from modified waxy rice starches: nutritional aspects and textural characteristics

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ABSTRACT

Experimental gluten-free (GF) rice cookies were formulated with 100% rice flour (CTR) or by substituting 50 % of rice flour with native waxy rice starch (WRS) or with three different resistant starch (RS) ingredients obtained from debranched, annealed or acid and heat-moisture treated WRS (RS_a, RS_b and RS_c, respectively). Chemical composition, *in vitro* starch digestibility and physical and textural characteristics were carried out. Among cookies, RS_a-cookies had the highest total dietary fibre content, the lowest rapidly digestible starch and the highest RS contents. All the three RS preparations have proved effective in increasing the proportion that tested as RS with respect to native WRS. However, the estimated RS loss for each applied RS ingredients caused by the baking process followed the order of $RS_a < RS_c < RS_b$. Last, the lowest *vitro* glycaemic index value was measured for RS_a-cookies. Among cookies, differences in colour and hardness were reported. The partial replacement of commercial rice flour with RS_a could contribute to formulate GF cookies with higher dietary fibre content and likely slowly digestible starch properties more than equivalent amounts of RS_b and RS_c.

Keywords: Gluten-free; Resistant starch; Predicted glycemic index; Cookie.

1. Introduction

Coeliac disease is considered one of the most common food induced enteropathy caused by the ingestion of gluten containing grains in genetically susceptible individuals and, till date, the only successful treatment for coeliac affected patients is a lifelong adherence to a gluten-free (GF) diet (Pellegrini and Agostoni, 2015). However, indications suggested that several GF-rendered foods exhibit lower nutritional quality than their gluten containing counterparts, relatively higher total digestible carbohydrates and saturated fats and lower dietary fibre, protein and resistant starch (RS) contents being often reported comparing GF products to their gluten containing equivalents (Pellegrini and Agostoni, 2015; Foschia et al., 2017). In particular, the RS fraction has attracted the interest of nutritionists and food processors because of its potential physiological benefits. The RS fraction represents a particular form of starch able to reach the large intestine of human subject mainly undigested where can be fermented by gut microbiota favouring butyrate production (Raigond et al., 2015). There is ample justification through nutritional studies that RS consumption has the potential to promote hypoglycaemic effects, prevention of colorectal cancer, lower plasma cholesterol and triglyceride concentrations, inhibition of fat accumulation and an enhanced vitamin and mineral absorptions (Raigond et al., 2015). Accordingly, in order to bear aforementioned health claims, international dietary guidelines suggest that starch-baked foods should contain at least 14 % of RS on total starch (EFSA 2011).

Extensive research has been therefore conducted to investigate the preparation of a new generation of cereal-based GF foods formulated with high-RS sources as value-enriched ingredients (Foschia et al., 2017). One of the most common approaches is based on the partial replacement of digestible starch with RS ingredients derived from high amylose starch (HAS), either in the native form or after modification through hydrothermal, enzymatic and/or chemical treatments (Haralampu, 2000). Besides HAS, analogous processing schemes have been applied in different granular starches prior to food inclusion in an effort to enhance the proportion that tests as RS (Thompson, 2000). Accordingly, the interest in the preparation of value-added RS ingredients starting from waxy rice starch (WRS) is becoming more popular because of its wide-ranging food and industrial applications. Several studies revealed that higher amount of RS (from about +60 % to about +90 %) could be obtained from debranched WRS (Shi and Gao, 2011), from annealed WRS (Van Hung et al., 2016a) or from WRS subjected to a combination of acid and heat-moisture treatments when compared to native WRS (Van Hung et al., 2016b).

Even if promising results have been obtained, to the best of our knowledge no information is currently available concerning the utilization in baked GF products and the behaviour after cooking of high-RS ingredients obtained from WRS. A better understanding of their functionality issue would allow for the development of GF baked products with favourably improved nutritional value and starch digestion properties. Within this perspective, cookies, being one the largest categories of ready-to-eat foods worldwide consumed, could represent a potentially nutritious GF snack through the selection of ingredients (Sharma et al., 2016).

Therefore, the aim of this study was to evaluate if RS ingredients obtained from WRS could be advantageous to produce GF cookies with better nutritional qualities. Developed GF cookies were examined for nutritional composition, *in vitro* starch digestion properties and physical and textural characteristics that are considered important parameters in the formulation of related food products.

2. Materials & methods

2.1. Ingredients and resistant starch preparation

Commercial WRS (native waxy rice starch; 1.0-2.0 % amylose) was obtained from Riso Scotti SpA (Pavia, Italy). All other components (food grade) were acquired in local supermarkets and stored depends on individual requirements. All the chemicals and reagents were all of analytical grade.

Three distinct RS preparations were conducted, by subjecting native WRS to hydrolysis by pullulanase debranching enzyme, annealing and a combination of acid and heat-moisture treatments, respectively. The debranched treatment was based on the protocol detailed by Shi and Gao (2011). A WRS slurry (10 % w/w in diluted pH 4.5 buffer solution containing 0.2 M acetic acid and 0.2 M sodium acetate) was cooked at 95 °C for 30 min and then cooled to 58 °C. Then, 55 ASPU of heat-stable pullulanase (Diazyme® P10, 1000 ASPU/g, 1.15 g/ml; Danisco Company, USA) for each g of dry starch was added. The ASPU is defined as the amount of enzyme that liberates 1.0 mg of glucose from starch in 1 min at pH 4.4 and 60 °C. The slurry was re-incubated in a water bath at 58 °C for 12 h. After the reaction, the solution was heated at 100 °C for 30 min to stop the reaction and then cooled to room temperature for 24 h. The precipitated debranched starch residue was oven dried at 40 °C to a moisture content of about 9-10 %. For the annealing treatment, native WRS was mixed with distilled water at a ratio of 1:2 (w/w) in a sealed container and heated in a water bath at 45 °C for 24 h (Van Hung et al., 2016a). After incubation, the starch sample was dried as previously described. For the third preparation, native WRS was dispersed in a measured volume of 0.2 M citric acid solution with moisture level adjusted to 30 % in a sealed container (Van Hung et al., 2016b). After equilibration at room temperature for 24 h, the starch sample was heated at 110 °C for 8 h, neutralized with 1 M sodium hydroxide and then washed thoroughly with distilled water. The treated starch was recovered by centrifugation and then dried as previously reported. All resulting RS ingredients were finely ground (1-mm screen; Retsch ZM1; Brinkman Instruments, Rexdale, ON, Canada) and stored at room temperature. Hereafter, RS_a, RS_b and RS_c indicate the three different RS ingredients derived from debranched, annealed and acid-heat-moisture treated WRS, respectively.

2.2. Characterization of native and treated waxy rice starches

The thermal properties of native WRS and debranched, annealed and acid-heat-moisture treated WRS (RS_a, RS_b and RS_c, respectively) were studied in duplicate by differential scanning calorimetry (DSC) (DSC8000, Perkin Elmer Inc., USA) as detailed by Shi and Gao (2011). Briefly, samples (suspension of 30 % w/w solid:water) were heated from 30 °C to 150 °C at 10 °C/min. Parameters of interest were the onset (T_0), peak (T_p), the conclusion (T_c) temperatures and the enthalpy of gelatinization (ΔH).

The pasting properties were determined using the Rapid Viscoanalyzer (RVA-4500, Perten, Sweden) according to the approved method AACC (76-21.01) (AACC 2000). An aliquot of starch (3.0 g) was dispersed in distilled water (25 ml), scaling both sample and water weight on a 14 % (w/w) sample moisture basis. The suspension was subjected to the following temperature profile: holding at 50 °C for 1 min; heating from 50 to 95 °C; holding at 95 °C for 7.5 min; cooling from 95 °C to 50 °C; holding at 50 °C for 2 min. A heating/cooling rate of 6 °C/min was applied. Measurements were performed in duplicate and the average curve was reported. The water absorption capacity (WAC, %) was determined in duplicate following the procedure as reported by Dundar and Gocmen (2013).

2.3. Experimental gluten free rice cookie formulation and preparation

Five different GF rice cookies were prepared. For GF control cookies (CTR-cookies), the recipe was based on commercial rice flour (120 g), whole egg (80 g), distilled water (30 g), unsalted butter (20 g), salt (1.0 g) and sodium bicarbonate (1.0 g). For experimental GF rice cookies, part of rice flour equivalent to 50 % was replaced with the previously obtained RS ingredients to formulate RS_a-, RS_b- and RS_c-cookies, respectively. In addition, WRS-cookies were prepared, by replacing 50 % of rice flour with native WRS. For all formulations, no sugars were added to limit the amount of glycaemic carbohydrates. Briefly, butter was creamed, mixed with liquid ingredients and then added to dry ingredients. Materials were combined with a domestic blender (Kitchen Aid, Model K5SSWH, St. Joseph, Mich., U.S.A.) for 5 min to obtain homogeneous dough. The dough was laminated by a pasta roller attachment at 0.4 cm height, allowed to rest for 30 min at 4°C, cut with a circular mould (4 cm diameter) and baked using a household oven (RKK 66130, Rex International, Italy) at a temperature of 180 ± 4 °C for 20 ± 2 min. Once baked, all GF cookies (i.e., CTR-, WRS-, RS_a-, RS_b- and RS_c-cookies) were cooled and kept in separate airtight plastic bags at room temperature until analysis. For each recipe, three batch replicates were produced on the same day.

2.4. Chemical composition of gluten free rice cookies

Cookie samples were dried at 55 °C for 24 h in a forced-air oven and ground through a 1-mm screen using a laboratory mill (Retsch grinder model ZM1; Brinkman Instruments, Rexdale, ON, Canada). Analyses were performed according to AOAC (2000) for dry matter (DM; method 930.15), ash (method 942.05), crude protein (method 976.05) and crude lipid (method 954.02 without acid hydrolysis) contents. Enzymatic quantifications of total dietary fibre (Megazyme assay kit K-INTDF 02/15, which includes RS and non-digestible oligosaccharides as a component of total dietary fibre), total starch (Megazyme assay kit K-TSTA 07/11) and free sugars (Megazyme assay kit K-SUFRG 06/14) were carried out. For each treatment, batches were analyzed in triplicate.

2.5. Starch fraction contents, in vitro starch digestion and calculations

Dietary starch fraction content as rapidly digestible starch (RDS), slowly digestible starch (SDS) and RS was determined by controlled enzymatic hydrolysis (Englyst et al., 1992). The value for RDS was obtained from the glucose released after 20 min incubation. The value of

SDS was obtained as the glucose released after a further 100 min incubation whereas RS content (both for starch ingredients and GF cookies) was determined as the starch that remained un-hydrolysed after 120 min. The RS content of starch ingredients was: 15.2 g/100 g DM for commercial rice flour, 13.2 g/100g DM for native WRS, 71.4 g/100g DM for RS_a, 65.2 g/100g DM for RS_b and 50.4 g/100g DM for RS_c. Considering the RS content of each starch ingredient and its percentage into the corresponding GF cookie recipe, the estimated RS loss due to the baking process (% DM) for each ingredient was calculated using the expected *versus* the effectively measured RS content of corresponding GF cookies after correction for the amount of RS coming from commercial rice flour. The latter was calculated taking into account the RS content of CTR-cookies after the baking process.

The multi-enzymatic protocol detailed by Giuberti et al. (2015a) was employed to evaluate the starch hydrolysis potential of samples "as eaten". Cookies were cut into homogeneous small pieces through a mortar to simulate mastication. Thereafter, samples (800 mg of available starch) were weighed accordingly in 50 ml test tubes and pre-treated with a 0.05 M HCl solution containing pepsin (5 mg/ml; P-7000, Sigma-Aldrich® Co., Milan, Italy) for 30 min at 37° C under gentle agitation. To all tubes, five glass balls were added to enhance agitation and provide a mechanical disruption of samples. The pH of the solution was then adjusted to 5.2 by adding 0.1 M sodium acetate buffer prior to the addition of an enzyme mixture with an amylase activity of about 7000 U/mL (Englyst et al., 1992) given by pancreatin (about 7500 FIP-U/g; 7130, Merck KGaA, Darmstadt, Germany), amyloglucosidase (about 300 U/ml; A-7095, Sigma-Aldrich® Co., Milan, Italy) and invertase (about 300 U/g; I-4504, Sigma-Aldrich® Co., Milan, Italy). Aliquots were carefully taken from each tube at 0 (prior to the addition of the enzyme mixture simulating the pancreatic phase), 15, 30, 60, 90, 120 and at 180 min after the enzyme addition, absolute ethanol was added and the amount of released glucose was determined colorimetrically with a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic snc, Rome, Italy). A blank was also included to correct for the glucose present in amyloglucosidase solution. The percentage of hydrolysed starch at each time interval was calculated using a factor of 0.9. Batches were analyzed in triplicate.

A hydrolysis index (HI) was then derived from the ratio between the area under the hydrolysis curve (0-180 min) of each cookie and the corresponding area of a reference sample (commercial fresh white wheat bread; WWB) as a percentage over the same period. From the HI, an *in vitro* GI value was derived using the formula: *in vitro* GI = 0.862 x HI + 8.198 (Granfeldt, 1994).

To describe starch hydrolysis kinetics, a first-order exponential model with the form $C_t = C_0 + C_\infty (1 - e^{-kt})$ was applied (Giuberti et al., 2012). In particular, C_t was the starch hydrolysed at time t (g/100 g dry starch), C_0 was the starch solubilized in the buffer at 0 min (g/100 g dry starch), C_∞ was the equilibrium concentration (g/100 g dry starch), k was the hydrolysis rate constant (min^{-1}) and t was the incubation time (min). For the purpose of data fitting, values were obtained by the Marquardt method using the PROC NLIN procedure of SAS 9.3 (SAS Inst. Inc., Cary, N.C., U.S.A).

2.6. Physical and textural characteristics of gluten free rice cookies

Diameter and thickness of cookies were determined with a Vanier calliper at three different points. The spread ratio was calculated a reported by Sharma et al. (2016), whereas the colour of GF cookies was measured on the basis of CIE *L** (lightness), *a** (redness-greenness) and *b** (yellowness-blueness) colour system using a Minolta CR410 Chroma Meter (Konica Minolta Co., Japan). For each batch, 5 readings were taken.

Hardness analysis was performed with a TA-XT2i Texture Analyser (Stable Micro Systems, UK) fitted with a shape blade-cutting probe. The crosshead speed was 10 mm/s, data were acquired with a resolution of 500 Hz and a 5 kg load cell was used. For each batch, five cookies were tested. Texture Export Exceed Release 2.54 (Stable Micro System) was then used to acquire the maximum peak force to snap cookies (hardness) expressed as fracture force (*N*) (Sharma et al., 2016).

2.7. Statistical analyses

Normal distribution of data was verified by the Shapiro-Wilk test before statistical analysis. Data were analyzed as a completely randomised design using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, N.C., USA) according to the model: $Y_{ij} = \mu + \alpha_i + e_{ij}$, where Y_{ij} is the dependent variable of the jth subject (GF cookie batch) assigned to treatment i, μ is the overall mean, α_i is the fixed effect of treatments (i = 5, being CTR, WRS, RS_a, RS_b and RS_c cookies or single ingredients), and e_{ij} is the residual error. Experimental unit was the GF cookie batch. Significance was declared at $p < 0.05$.

3. Results and discussion

3.1. Characterization of native and treated waxy rice starches

Thermal properties of starch samples are descriptively presented in Table 1. Compared to native WRS, all the three RS preparations showed an increase in the transition temperatures (T_0, T_p and T_c) and in ΔH values, in line with previous findings (Shi and Gao 2011; Zeng et al., 2015). The increase in the transition temperatures can indicate a formation of intermolecular hydrogen bond, improved crystalline perfection and a more intense interaction between starch molecules, while higher ΔH values can be related to differences in bonding forces between the double helices that form amylopectin crystallites, which resulted in different alignment of hydrogen bonds within starch molecules (Hoover, 2010; Pratiwi et al., 2017).

Pasting properties of native WRS and RS preparations are given in Fig. 1. The WRS exhibited a sharp increase in viscosity, reaching the peak viscosity in a short time (*e.g.* low temperatures), which is typical of WRS (Shih et al., 2007). The profile showed high breakdown (2599 cP), high setback (729 cP) and low final (1878 cP) viscosities in the cooling phase at the end of the temperature program cycle. Annealing (RS_b) caused a decrease in peak (2442 cP), trough (762 cP), breakdown (1680 cP), final (1068 cP) and setback (306 cP) viscosities, with little influence on peak time and pasting temperature. This reduction might be due to the disrupted starch granules and partial solubilization caused by the annealing process. Previous studies reported that annealing altered the RVA pasting properties of starches from various botanical sources such as wheat, potato, and pea, but it had only limited effect on rice starch (Jacobs et al., 1995). However, changes in pasting profile after annealing strongly depended on the botanical source of starch, method and annealing conditions applied. Compared to the RS_b, both RS_a and RS_c exhibited significant differences in their behaviour during heating and cooling in excess of water, as a consequence of a different rearrangement of the granular architecture in the treated samples. Both RS_a and RS_c samples did not develop pasting viscosities under the experimental conditions. Enzymatic hydrolysis with pullulanase (as in RS_a) might have increased formation of short linear chain molecules and RS content which could lead to a decrease in pasting viscosity along with a reduced ability of forming gel (Polesi and Sarmento, 2011; Reddy et al., 2015). Considering RS_c, the citric acid and heat treatments were reported to change the internal structure and physicochemical properties of starch such as producing more various short chains, forming different crystallites with different melting temperatures, viscosity and gel-forming ability (Shin et al., 2007; Van Hung et al., 2016).

Overall, present findings indicated that both debranching, annealing and heat-moisture treatments altered the internal rearrangement of native WRS granules to different extents. In particular, RS_a and RS_c samples would behave differently from WRS and RS_b during cooking and processing, likely remaining unchanged under most food processing conditions (Lei et al., 2008). Moreover, results suggested that RS_a and RS_c might withstand hydrolysis by human digestive enzymes (Lei et al., 2008; Pratiwi et al., 2017).

Last, different WAC values were measured comparing RS_a, RS_b and RS_c to native WRS. Similar effect on WAC values has been reported as due heat-moisture treatment of high amylose maize starch (Dundar and Gocmen, 2013). Present findings may be related to the difference in the degree of availability of water bindings sites in the different samples, which strongly depends on the ultra-structural and compositional differences of selected starches (Dundar and Gocmen, 2013).

3.2. Chemical composition of gluten-free rice cookies

The nutrient composition of experimental GF rice cookies (Table 2) appears in line with previous findings (Giuberti et al., 2015b). Differences ($p < 0.05$) among samples were reported for total starch, crude protein and ash contents. In particular, RS_a- and RS_c-cookies had the lowest total starch content (on average 56.4 g/100g DM; $p < 0.05$), whereas an average lower crude protein content was measured for WRS-, RS_a-, RS_b- and RS_c-cookies when compared to CTR-cookies (13.0 *versus* 15.6 g/100g DM, respectively; $p < 0.05$). Differences in the chemical composition have already been obtained in RS-enriched pasta compared to the control (Bustos et al., 2011). In addition, the partial replacement of rice flour with the applied RS ingredients caused a significant rise in the total dietary fibre content, the highest value obtained for RS_a-cookies (15.1 g/100g DM; $p < 0.05$). An enhanced total dietary fibre contents has been reported in wheat pasta and in GF bread samples formulated with different RS sources (Gelencsér et al., 2010; Giuberti et al., 2016). From a nutritional standpoint, to claim that a food is a "source of dietary fibre", it should contain at least 3 g per

100 g of serving of total dietary fibre, whereas the claim ‘high in dietary fibre” is assigned to food with at least 6 g/100g. Therefore, RS_a- and RS_c-cookies can be considered high dietary fibre food products. Greater amounts of dietary fibre by GF baked products are considered beneficial, since a general low intake of this food component has been described for the coeliac population (Pellegrini and Agostoni, 2015). No differences were reported for crude lipid and free sugar contents, on average being 13.2 g/100g DM and 0.2 g/100g DM, respectively. Average moisture content of 3.2 g/100g cookies was reported, thus indicating a long shelf life of the products (Giuberti et al., 2015b).

3.3. Starch fractions of gluten-free cookies and estimated resistant starch loss of ingredients

As reported in Table 3, values of 31.5, 29.4 and 1.5 g/100g DM were respectively measured for RDS, SDS and RS in CTR-cookies, appearing in line with our previous findings obtained for GF cookies formulated with 100 % GF commercial flour blend (Giuberti et al., 2015b). In addition, compared to CTR-, WRS-cookies were characterized by higher RDS ($p < 0.05$) and a numerically lower RS contents. Data from literature suggest that higher RDS and lower RS contents generally characterized foods containing waxy and/or low-amylose starches when compared to foods formulated with normal amylose starches, being recognized that amylopectin possesses a much larger surface area per molecule than amylose, which makes it a preferable substrate for amylolytic attack (Singh et al., 2010). In all cases, the replacement of a part of commercial rice flour with the three different RS ingredients influenced the starch fraction contents in different ways, indicating that the behaviour of the applied RS ingredients changed during the baking process (Table 3). In particular, RS_a-cookies had the lowest RDS and the highest RS contents (25.6 and 13.3 g/100g DM, respectively; $p < 0.05$), whereas the lowest SDS content was measured in RS_b-cookies (13.0 g/100 g DM; $p < 0.05$). Similar changes have already been reported in GF breads (Giuberti et al., 2016) and in wheat pasta (Bustos et al., 2011; Aravind et al., 2013) formulated with different RS preparations. From a nutritional standpoint, there is general consensus that RDS ingestion promotes a fast increase in blood glucose and insulin levels in human subjects, whereas SDS usually provides a slow and prolonged release of glucose into the blood stream (Raigond et al., 2015). In addition, both RS_a- and RS_c-cookies contained more than 14 % of RS on a total dry starch basis, thus supporting international health claim recommendations (EFSA, 2011).

In the current evaluation, all the three RS preparations have proved effective in increasing the proportion that tested as RS, in line with previous findings (Shi and Gao, 2011; Van Hung et al., 2016a, 2016b). In particular, compared to the RS content of native WRS (13.2 g/100g DM), all obtained RS ingredients had greater RS yield, with values of 71.4 g/100g DM for RS_a (debranched WRS), 65.2 g/100 g DM for RS_b (annealed WRS) and 50.4 g/100g DM for RS_c (acid-heat-moisture treated WRS). Usually, the term heat-moisture treatment is used when low moisture levels (< 35 % w/w) are applied, whereas, annealing refers to treatment of starch in excess (< 65 % w/w) or at intermediate (40-55 % w/w) water levels (Hoover, 2010). As a function of the starting materials and the applied protocols, annealing and heat-moisture treatments can result in structural changes within the amorphous and crystalline regions of starch to different extent, which in turn can influence enzyme susceptibility by either improve the order of the crystalline fraction or enhance the proportion of this fraction (Thompson, 2000). In addition, a limited acid hydrolysis prior to hydrothermal treatments can contribute to the formation of starch resistant to digestion, due to the presence of either short linear chains with enhanced mobility or cross-linking structures between starch chains that appear to participate in the formation of resistant portions through rearrangement and recrystallization of starch during subsequent cooling (Thompson, 2000). Last, since amylopectin chains can interfere with amylose retrogradation, cutting of amylopectin into shorter starch chains with debranching enzyme such as pullulanase can further increase the RS yield (Haralampu, 2000).

The RS content of both raw commercial rice flour and native WRS markedly decreased during the baking process, with estimated RS loss values closer to 90 % (Table 3). Due to the heating of processing, it can be expected that the RS content of raw ingredients will be significantly reduced by disrupting the semicrystalline structure of starch granules during the gelatinization process (Vasanthan and Bhatty, 1998). In addition, RS loss values of 49.5 %, 58.8 % and 90.5 % were estimated for RS_a-, RS_c- and RS_b-ingredients, respectively ($p < 0.05$), thus indicating a different thermal behavior of the applied RS ingredients. Based on these values, we can therefore suppose a heat stability in the order of RS_a (debranched WRS) > RS_c (acid and heat moisture treated WRS) > RS_b (annealed WRS). Despite it has been reported that the annealing treatment of WRS can result in structural changes within the amorphous and crystalline regions that may lead to the formation of a thermo-stable RS complex (Van Hung et al., 2016a), in our experimental conditions RS_b ingredient markedly lost its thermal stability during subsequent baking to an higher extent with respect to RS_a and RS_c ingredients. It is difficult to acquire a consensus on the effect of annealing from literature due to difference in the preparation conditions, starch sources and applied digestion protocols (Hoover, 2010). In addition, high-RS ingredients from WRS have been only analyzed immediately after their preparation, but never after their incorporation into food and the subsequent cooking process. However, Zeng et al. (2015) showed a lesser RS content in WRS subjected to dual hydrothermal treatment (combination of annealing and heat-moisture treatment) when compared to native WRS or to WRS subjected only to annealing treatment. Authors (Zeng et al., 2015) attributed these findings to an increase in starch granule porosity (facilitating enzyme activities) and/or a disruption in those crystallites that were perfect after the single hydrothermal treatment, thus leading to a RS loss during the subsequent heat treatment.

In addition, present findings suggested that treating WRS with pullulanase debranching enzyme prior to the heat treatment may contribute to create more ordered crystalline structures with enough heat stability to maintain their close packing under cooking conditions (Vasanthan and Bhatty, 1998). During debranching, WRS would release relatively short linear fragments similar to amylose that could re-associate leading to a new and strong crystalline structure upon cooking, thereby leading to the formation of a more stable RS complex (Guraya et al., 2001). Likewise, the inclusion of 20 % of RS obtained from debranched HAS from maize contributed to formulate GF-breads with higher RS content more than equivalent amounts of HAS maize subjected to three consecutive autoclaving-cooling cycles, even if RS losses for single ingredients were not reported (Giuberti et al., 2016). In addition, Shi and Gao (2011) reported an increase in the apparent amylose content in the debranched WRS with respect to native WRS. The presence of amylose can affect the RS formation, by reducing the degree of starch swelling during gelatinization and/or by leading to a tightly packed crystalline structure during starch retrogradation on cooling (Haralampu, 2000).

Up to now, no information is present on the RS loss of aforementioned RS ingredients obtained from WRS subjected to a subsequent cooking process after incorporation into cookies. For other food categories and RS sources, contrasting results have been reported. In particular, Gelencsér et al. (2010) found a decreased RS content after cooking (on average -50 %) in RS-enriched wheat pasta samples formulated with RS from HAS of from chemically modified phosphate starch. In contrast, Aravind et al. (2013) did not report changes caused by processing comparing uncooked and cooked pasta samples containing RS from native or from retrograded HAS. Also Aparacio-Saguilán et al. (2007) pointed out to a similar result using

RS from lintnerized banana starch in wheat cookies. Differences in experimental conditions, RS sources and preparations along with different method used for RS determination could explain these discrepancies. Further investigations concerning the relationship between heat stability of various RS formulations and the baking process are therefore required to maximize RS content in the eaten products.

3.4. In vitro glycaemic index of gluten-free cookies

The GI concept has been introduced to classify different carbohydrate-rich foods with respect to their effect on post-meal glycaemia. Accordingly, foods can be classified into three main categories, having low (<55), medium (55–69) and high (>70) GI (Foster-Powell et al., 2002). Nowadays, there is considerable interest in lowering the GI of high digestible foods since a long-term intake of lower-GI foods may favourably influence post-prandial and insulin responses and can be beneficial for prevention and control of obesity and metabolic risk factors (Raigond et al., 2015). Both *in vivo* and *in vitro* methods have been developed to allow the evaluation of GI values and *in vitro* digestion models can represent a viable, rapid and cost effective alternative for the prediction of the *in vivo* GI and for a preliminary screening of new-developed products.

Using WWB as reference, CTR-cookies were characterized by an *in vitro* GI value of 90, in line with previous indications for analogous GF food categories (Foster-Powell et al., 2002) (Table 4). The incorporation of RS ingredients reduced to a different extent the *in vitro* GI of cookies with respect to CTR- and WRS-cookies, the lowest value recorded for RS_a-cookies (i.e., 71; $p < 0.05$). Present findings could be related to the respective RDS and RS contents of individual GF cookie categories, these fractions being respectively related in positive and negative ways to *in vitro* GI values (Giuberti et al., 2012; Aravind et al., 2013). In addition, the increasing amount of total dietary fibre found in RS-enriched GF cookies, along with the possible formation of amylose-lipid complexes during cooking, could have contributed to further reduce the accessibility of amylase to hydrolyse the starch (Singh et al., 2010). Last, different ($p < 0.05$) k values, which reflect the rate of starch hydrolysis, were obtained (Table 4). In particular, RS_a- and RS_c-cookies had the lowest ($p < 0.05$) k values when compared to all other cookies, being 0.017 min^{-1} and 0.022 min^{-1}, respectively. This indicates that starch contained in RS_a- and RS_c-cookies was less susceptible to the digestive enzymes and much slower hydrolysed than starch contained in CTR-, WRS- and RS_b-cookies. The consumption of foods with slowly digestible starch properties may be beneficial for the prevention of hyperglycaemia-related disorders, such as diabetes and cardiovascular diseases (Raigond et al., 2015). However, in order to confirm present *in vitro* evaluations, *in vivo* results are strongly recommended.

3.5. Physical and textural characteristics of gluten free rice cookies

The results of various physical and textural characteristics are shown in Table 5. Significant differences among cookies ($p < 0.05$) were observed in the colour and hardness parameters. In particular, RS_b-cookies displayed the highest L^* and b^* (77.0 and 38.3, respectively; $p < 005$) and the lowest a^* values (1.2; $p < 0.05$). These difference can be related to uneven exposure of cookies' surface area to baking temperature, thus leading to different chemical reactions such us Maillard reactions which occur during baking (Uthumporn et al., 2015). In addition, RS_a-cookies were the hardest in texture, being 67.9 *N* ($p < 0.05$). It is well recognized that hardness of cookies is much affected by the composition of flours and interaction among ingredients. In particular, Norhidayah et al. (2014) reported the highest hardness value for cookies with higher amounts of RS. Presumably, some of the starch granules remained in their native form during baking and did not form a continuous structure, thus leading to an increase in hardness (Norhidayah et al. (2014). In addition, the higher dietary fibre content of RS_a-cookies could have contributed to further increase this value, as already reported in cookies made with eggplant flour (Uthumporn et al., 2015). Last, similar diameter, thickness and spread ratio values were obtained. Cookie spread represents a ratio of diameter and height and, in general, cookies with higher spread ratio are considered the most desirable. Slightly higher, but still comparable, spread ratio values (on average 5.6) have been reported for GF cookies made from flour blends of minor millets (Sharma et al., 2016).

4. Conclusions

Five different GF-cookies were formulated using 100 % rice flour or blends with 50:50 rice flour and native WRS or three different RS ingredients obtained by subjecting WRS to hydrolysis by pullulanase debranching enzyme (RS_a), annealing (RS_b) and a combination of acid and heat-moisture treatments (RS_c). Both thermal and pasting properties differed among starch ingredients. Considering GF-cookies, differences in the chemical composition and in the *in vitro* starch digestion characteristics were reported. In addition, despite all the three RS preparations have proved effective in increasing the total amount of RS, analyses revealed that the heat stability of these RS ingredients decreased in the order of $RS_a > RS_c > RS_b$. Consequently, the higher RS content, along with the lower *in vitro* GI values, were obtained for RS_a-cookies. Among cookies, similar diameter, thickness and spread ratio values were measured, whereas significant differences in colour and hardness were reported. Taking together, present *in vitro* findings suggested that the partial replacement of rice flour with a RS ingredient obtained through debranching WRS could contributed to formulate GF rice cookies with likely slowly digestible starch properties more than equivalent amounts of RS ingredients obtained by subjecting WRS to annealing or acid-heat moisture treatments. Present *in vitro* findings would help to better understand the properties of modified WRS as a potentially source of RS in baked GF products. However, in order to confirm present *in vitro* results, *in vivo* trials are strongly warranted.

Acknowledgments

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REVISION CHECKLIST

In the revised version, the following analyses were added:

1- DSC analysis

2- RVA analysis

3-Water absorption capacity analysis

Description of analyses was inserted from lines 139 to 154.

A new table (table 1) and a new figure (figure 1) are now present containing parameters of interest.

Additional results are discussed in lines 254-298.

The revised version contains:

- Manuscript
- 5 Tables
- 1 Figure

Highlights:

- Waxy rice starch was modified to enhance its resistant starch content.
- Gluten free cookies were studied considering nutritional aspects and textural characteristics.
- Difference in the starch fraction contents and the *in vitro* glycaemic index values were obtained.
- Debranched waxy rice starch had greater thermal stability.
- Different colour and hardness values were obtained among samples.

Gluten free rice cookies with resistant starch ingredients from modified waxy rice starches: nutritional aspects and textural characteristics

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ABSTRACT

Experimental gluten-free (GF) rice cookies were formulated with 100% rice flour (CTR) or by substituting 50 % of rice flour with native waxy rice starch (WRS) or with three different resistant starch (RS) ingredients obtained from debranched, annealed or acid and heat-moisture treated WRS (RS_a, RS_b and RS_c, respectively). Chemical composition, *in vitro* starch digestibility and physical and textural characteristics were carried out. Among cookies, RS_a-cookies had the highest total dietary fibre content, the lowest rapidly digestible starch and the highest RS contents. All the three RS preparations have proved effective in increasing the proportion that tested as RS with respect to native WRS. However, the estimated RS loss for each applied RS ingredients caused by the baking process followed the order of $RS_a < RS_c < RS_b$. Last, the lowest *vitro* glycaemic index value was measured for RS_a-cookies. Among cookies, differences in colour and hardness were reported. The partial replacement of commercial rice flour with RS_a could contribute to formulate GF cookies with higher dietary fibre content and likely slowly digestible starch properties more than equivalent amounts of RS_b and RS_c.

Keywords: Gluten-free; Resistant starch; Predicted glycemic index; Cookie.

1. Introduction

Coeliac disease is considered one of the most common food induced enteropathy caused by the ingestion of gluten containing grains in genetically susceptible individuals and, till date, the only successful treatment for coeliac affected patients is a lifelong adherence to a gluten-free (GF) diet (Pellegrini and Agostoni, 2015). However, indications suggested that several GF-rendered foods exhibit lower nutritional quality than their gluten containing counterparts, relatively higher total digestible carbohydrates and saturated fats and lower dietary fibre, protein and resistant starch (RS) contents being often reported comparing GF products to their gluten containing equivalents (Pellegrini and Agostoni, 2015; Foschia et al., 2017). In particular, the RS fraction has attracted the interest of nutritionists and food processors because of its potential physiological benefits. The RS fraction represents a particular form of starch able to reach the large intestine of human subject mainly undigested where can be fermented by gut microbiota favouring butyrate production (Raigond et al., 2015). There is ample justification through nutritional studies that RS consumption has the potential to promote hypoglycaemic effects, prevention of colorectal cancer, lower plasma cholesterol and triglyceride concentrations, inhibition of fat accumulation and an enhanced vitamin and mineral absorptions (Raigond et al., 2015). Accordingly, in order to bear aforementioned health claims, international dietary guidelines suggest that starch-baked foods should contain at least 14 % of RS on total starch (EFSA 2011).

Extensive research has been therefore conducted to investigate the preparation of a new generation of cereal-based GF foods formulated with high-RS sources as value-enriched ingredients (Foschia et al., 2017). One of the most common approaches is based on the partial replacement of digestible starch with RS ingredients derived from high amylose starch (HAS), either in the native form or after modification through hydrothermal, enzymatic and/or chemical treatments (Haralampu, 2000). Besides HAS, analogous processing schemes have been applied in different granular starches prior to food inclusion in an effort to enhance the proportion that tests as RS (Thompson, 2000). Accordingly, the interest in the preparation of value-added RS ingredients starting from waxy rice starch (WRS) is becoming more popular because of its wide-ranging food and industrial applications. Several studies revealed that higher amount of RS (from about +60 % to about +90 %) could be obtained from debranched WRS (Shi and Gao, 2011), from annealed WRS (Van Hung et al., 2016a) or from WRS subjected to a combination of acid and heat-moisture treatments when compared to native WRS (Van Hung et al., 2016b).

Even if promising results have been obtained, to the best of our knowledge no information is currently available concerning the utilization in baked GF products and the behaviour after cooking of high-RS ingredients obtained from WRS. A better understanding of their functionality issue would allow for the development of GF baked products with favourably improved nutritional value and starch digestion properties. Within this perspective, cookies, being one the largest categories of ready-to-eat foods worldwide consumed, could represent a potentially nutritious GF snack through the selection of ingredients (Sharma et al., 2016).

Therefore, the aim of this study was to evaluate if RS ingredients obtained from WRS could be advantageous to produce GF cookies with better nutritional qualities. Developed GF cookies were examined for nutritional composition, *in vitro* starch digestion properties and physical and textural characteristics that are considered important parameters in the formulation of related food products.

2. Materials & methods

2.1. Ingredients and resistant starch preparation

Commercial WRS (native waxy rice starch; 1.0-2.0 % amylose) was obtained from Riso Scotti SpA (Pavia, Italy). All other components (food grade) were acquired in local supermarkets and stored depends on individual requirements. All the chemicals and reagents were all of analytical grade.

Three distinct RS preparations were conducted, by subjecting native WRS to hydrolysis by pullulanase debranching enzyme, annealing and a combination of acid and heat-moisture treatments, respectively. The debranched treatment was based on the protocol detailed by Shi and Gao (2011). A WRS slurry (10 % w/w in diluted pH 4.5 buffer solution containing 0.2 M acetic acid and 0.2 M sodium acetate) was cooked at 95 °C for 30 min and then cooled to 58 °C. Then, 55 ASPU of heat-stable pullulanase (Diazyme® P10, 1000 ASPU/g, 1.15 g/ml; Danisco Company, USA) for each g of dry starch was added. The ASPU is defined as the amount of enzyme that liberates 1.0 mg of glucose from starch in 1 min at pH 4.4 and 60 °C. The slurry was re-incubated in a water bath at 58 °C for 12 h. After the reaction, the solution was heated at 100 °C for 30 min to stop the reaction and then cooled to room temperature for 24 h. The precipitated debranched starch residue was oven dried at 40 °C to a moisture content of about 9-10 %. For the annealing treatment, native WRS was mixed with distilled water at a ratio of 1:2 (w/w) in a sealed container and heated in a water bath at 45 °C for 24 h (Van Hung et al., 2016a). After incubation, the starch sample was dried as previously described. For the third preparation, native WRS was dispersed in a measured volume of 0.2 M citric acid solution with moisture level adjusted to 30 % in a sealed container (Van Hung et al., 2016b). After equilibration at room temperature for 24 h, the starch sample was heated at 110 °C for 8 h, neutralized with 1 M sodium hydroxide and then washed thoroughly with distilled water. The treated starch was recovered by centrifugation and then dried as previously reported. All resulting RS ingredients were finely ground (1-mm screen; Retsch ZM1; Brinkman Instruments, Rexdale, ON, Canada) and stored at room temperature. Hereafter, RS_a, RS_b and RS_c indicate the three different RS ingredients derived from debranched, annealed and acid-heat-moisture treated WRS, respectively.

2.2. Characterization of native and treated waxy rice starches

The thermal properties of native WRS and debranched, annealed and acid-heat-moisture treated WRS (RS_a, RS_b and RS_c, respectively) were studied in duplicate by differential scanning calorimetry (DSC) (DSC8000, Perkin Elmer Inc., USA) as detailed by Shi and Gao (2011). Briefly, samples (suspension of 30 % w/w solid:water) were heated from 30 °C to 150 °C at 10 °C/min. Parameters of interest were the onset (T_0), peak (T_p), the conclusion (T_c) temperatures and the enthalpy of gelatinization (ΔH).

The pasting properties were determined using the Rapid Viscoanalyzer (RVA-4500, Perten, Sweden) according to the approved method AACC (76-21.01) (AACC 2000). An aliquot of starch (3.0 g) was dispersed in distilled water (25 ml), scaling both sample and water weight on a 14 % (w/w) sample moisture basis. The suspension was subjected to the following temperature profile: holding at 50 °C for 1 min; heating from 50 to 95 °C; holding at 95 °C for 7.5 min; cooling from 95 °C to 50 °C; holding at 50 °C for 2 min. A heating/cooling rate of 6 °C/min was applied. Measurements were performed in duplicate and the average curve was reported. The water absorption capacity (WAC, %) was determined in duplicate following the procedure as reported by Dundar and Gocmen (2013).

2.3. Experimental gluten free rice cookie formulation and preparation

Five different GF rice cookies were prepared. For GF control cookies (CTR-cookies), the recipe was based on commercial rice flour (120 g), whole egg (80 g), distilled water (30 g), unsalted butter (20 g), salt (1.0 g) and sodium bicarbonate (1.0 g). For experimental GF rice cookies, part of rice flour equivalent to 50 % was replaced with the previously obtained RS ingredients to formulate RS_a-, RS_b- and RS_c-cookies, respectively. In addition, WRS-cookies were prepared, by replacing 50 % of rice flour with native WRS. For all formulations, no sugars were added to limit the amount of glycaemic carbohydrates. Briefly, butter was creamed, mixed with liquid ingredients and then added to dry ingredients. Materials were combined with a domestic blender (Kitchen Aid, Model K5SSWH, St. Joseph, Mich., U.S.A.) for 5 min to obtain homogeneous dough. The dough was laminated by a pasta roller attachment at 0.4 cm height, allowed to rest for 30 min at 4°C, cut with a circular mould (4 cm diameter) and baked using a household oven (RKK 66130, Rex International, Italy) at a temperature of 180 ± 4 °C for 20 ± 2 min. Once baked, all GF cookies (i.e., CTR-, WRS-, RS_a-, RS_b- and RS_c-cookies) were cooled and kept in separate airtight plastic bags at room temperature until analysis. For each recipe, three batch replicates were produced on the same day.

2.4. Chemical composition of gluten free rice cookies

Cookie samples were dried at 55 °C for 24 h in a forced-air oven and ground through a 1-mm screen using a laboratory mill (Retsch grinder model ZM1; Brinkman Instruments, Rexdale, ON, Canada). Analyses were performed according to AOAC (2000) for dry matter (DM; method 930.15), ash (method 942.05), crude protein (method 976.05) and crude lipid (method 954.02 without acid hydrolysis) contents. Enzymatic quantifications of total dietary fibre (Megazyme assay kit K-INTDF 02/15, which includes RS and non-digestible oligosaccharides as a component of total dietary fibre), total starch (Megazyme assay kit K-TSTA 07/11) and free sugars (Megazyme assay kit K-SUFRG 06/14) were carried out. For each treatment, batches were analyzed in triplicate.

2.5. Starch fraction contents, in vitro starch digestion and calculations

Dietary starch fraction content as rapidly digestible starch (RDS), slowly digestible starch (SDS) and RS was determined by controlled enzymatic hydrolysis (Englyst et al., 1992). The value for RDS was obtained from the glucose released after 20 min incubation. The value of

SDS was obtained as the glucose released after a further 100 min incubation whereas RS content (both for starch ingredients and GF cookies) was determined as the starch that remained un-hydrolysed after 120 min. The RS content of starch ingredients was: 15.2 g/100 g DM for commercial rice flour, 13.2 g/100g DM for native WRS, 71.4 g/100g DM for RS_a, 65.2 g/100g DM for RS_b and 50.4 g/100g DM for RS_c. Considering the RS content of each starch ingredient and its percentage into the corresponding GF cookie recipe, the estimated RS loss due to the baking process (% DM) for each ingredient was calculated using the expected *versus* the effectively measured RS content of corresponding GF cookies after correction for the amount of RS coming from commercial rice flour. The latter was calculated taking into account the RS content of CTR-cookies after the baking process.

The multi-enzymatic protocol detailed by Giuberti et al. (2015a) was employed to evaluate the starch hydrolysis potential of samples "as eaten". Cookies were cut into homogeneous small pieces through a mortar to simulate mastication. Thereafter, samples (800 mg of available starch) were weighed accordingly in 50 ml test tubes and pre-treated with a 0.05 M HCl solution containing pepsin (5 mg/ml; P-7000, Sigma-Aldrich® Co., Milan, Italy) for 30 min at 37° C under gentle agitation. To all tubes, five glass balls were added to enhance agitation and provide a mechanical disruption of samples. The pH of the solution was then adjusted to 5.2 by adding 0.1 M sodium acetate buffer prior to the addition of an enzyme mixture with an amylase activity of about 7000 U/mL (Englyst et al., 1992) given by pancreatin (about 7500 FIP-U/g; 7130, Merck KGaA, Darmstadt, Germany), amyloglucosidase (about 300 U/ml; A-7095, Sigma-Aldrich® Co., Milan, Italy) and invertase (about 300 U/g; I-4504, Sigma-Aldrich® Co., Milan, Italy). Aliquots were carefully taken from each tube at 0 (prior to the addition of the enzyme mixture simulating the pancreatic phase), 15, 30, 60, 90, 120 and at 180 min after the enzyme addition, absolute ethanol was added and the amount of released glucose was determined colorimetrically with a glucose oxidase kit (GODPOD 4058, Giesse Diagnostic snc, Rome, Italy). A blank was also included to correct for the glucose present in amyloglucosidase solution. The percentage of hydrolysed starch at each time interval was calculated using a factor of 0.9. Batches were analyzed in triplicate.

A hydrolysis index (HI) was then derived from the ratio between the area under the hydrolysis curve (0-180 min) of each cookie and the corresponding area of a reference sample (commercial fresh white wheat bread; WWB) as a percentage over the same period. From the HI, an *in vitro* GI value was derived using the formula: *in vitro* GI = 0.862 x HI + 8.198 (Granfeldt, 1994).

To describe starch hydrolysis kinetics, a first-order exponential model with the form $C_t = C_0 + C_\infty (1 - e^{-kt})$ was applied (Giuberti et al., 2012). In particular, C_t was the starch hydrolysed at time t (g/100 g dry starch), C_0 was the starch solubilized in the buffer at 0 min (g/100 g dry starch), C_∞ was the equilibrium concentration (g/100 g dry starch), k was the hydrolysis rate constant (min^{-1}) and t was the incubation time (min). For the purpose of data fitting, values were obtained by the Marquardt method using the PROC NLIN procedure of SAS 9.3 (SAS Inst. Inc., Cary, N.C., U.S.A).

2.6. Physical and textural characteristics of gluten free rice cookies

Diameter and thickness of cookies were determined with a Vanier calliper at three different points. The spread ratio was calculated a reported by Sharma et al. (2016), whereas the colour of GF cookies was measured on the basis of CIE L^* (lightness), a^* (redness-greenness) and b^* (yellowness-blueness) colour system using a Minolta CR410 Chroma Meter (Konica Minolta Co., Japan). For each batch, 5 readings were taken.

Hardness analysis was performed with a TA-XT2i Texture Analyser (Stable Micro Systems, UK) fitted with a shape blade-cutting probe. The crosshead speed was 10 mm/s, data were acquired with a resolution of 500 Hz and a 5 kg load cell was used. For each batch, five cookies were tested. Texture Export Exceed Release 2.54 (Stable Micro System) was then used to acquire the maximum peak force to snap cookies (hardness) expressed as fracture force (*N*) (Sharma et al., 2016).

2.7. Statistical analyses

Normal distribution of data was verified by the Shapiro-Wilk test before statistical analysis. Data were analyzed as a completely randomised design using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, N.C., USA) according to the model: $Y_{ij} = \mu + \alpha_i + e_{ij}$, where Y_{ij} is the dependent variable of the jth subject (GF cookie batch) assigned to treatment i, μ is the overall mean, α_i is the fixed effect of treatments (i = 5, being CTR, WRS, RS_a, RS_b and RS_c cookies or single ingredients), and e_{ij} is the residual error. Experimental unit was the GF cookie batch. Significance was declared at $p < 0.05$.

3. Results and discussion

3.1. Characterization of native and treated waxy rice starches

Thermal properties of starch samples are descriptively presented in Table 1. Compared to native WRS, all the three RS preparations showed an increase in the transition temperatures (T_0, T_p and T_c) and in ΔH values, in line with previous findings (Shi and Gao 2011; Zeng et al., 2015). The increase in the transition temperatures can indicate a formation of intermolecular hydrogen bond, improved crystalline perfection and a more intense interaction between starch molecules, while higher ΔH values can be related to differences in bonding forces between the double helices that form amylopectin crystallites, which resulted in different alignment of hydrogen bonds within starch molecules (Hoover, 2010; Pratiwi et al., 2017).

Pasting properties of native WRS and RS preparations are given in Fig. 1. The WRS exhibited a sharp increase in viscosity, reaching the peak viscosity in a short time (*e.g.* low temperatures), which is typical of WRS (Shih et al., 2007). The profile showed high breakdown (2599 cP), high setback (729 cP) and low final (1878 cP) viscosities in the cooling phase at the end of the temperature program cycle. Annealing (RS_b) caused a decrease in peak (2442 cP), trough (762 cP), breakdown (1680 cP), final (1068 cP) and setback (306 cP) viscosities, with little influence on peak time and pasting temperature. This reduction might be due to the disrupted starch granules and partial solubilization caused by the annealing process. Previous studies reported that annealing altered the RVA pasting properties of starches from various botanical sources such as wheat, potato, and pea, but it had only limited effect on rice starch (Jacobs et al., 1995). However, changes in pasting profile after annealing strongly depended on the botanical source of starch, method and annealing conditions applied. Compared to the RS_b, both RS_a and RS_c exhibited significant differences in their behaviour during heating and cooling in excess of water, as a consequence of a different rearrangement of the granular architecture in the treated samples. Both RS_a and RS_c samples did not develop pasting viscosities under the experimental conditions. Enzymatic hydrolysis with pullulanase (as in RS_a) might have increased formation of short linear chain molecules and RS content which could lead to a decrease in pasting viscosity along with a reduced ability of forming gel (Polesi and Sarmento, 2011; Reddy et al., 2015). Considering RS_c, the citric acid and heat treatments were reported to change the internal structure and physicochemical properties of starch such as producing more various short chains, forming different crystallites with different melting temperatures, viscosity and gel-forming ability (Shin et al., 2007; Van Hung et al., 2016).

Overall, present findings indicated that both debranching, annealing and heat-moisture treatments altered the internal rearrangement of native WRS granules to different extents. In particular, RS_a and RS_c samples would behave differently from WRS and RS_b during cooking and processing, likely remaining unchanged under most food processing conditions (Lei et al., 2008). Moreover, results suggested that RS_a and RS_c might withstand hydrolysis by human digestive enzymes (Lei et al., 2008; Pratiwi et al., 2017).

Last, different WAC values were measured comparing RS_a, RS_b and RS_c to native WRS. Similar effect on WAC values has been reported as due heat-moisture treatment of high amylose maize starch (Dundar and Gocmen, 2013). Present findings may be related to the difference in the degree of availability of water bindings sites in the different samples, which strongly depends on the ultra-structural and compositional differences of selected starches (Dundar and Gocmen, 2013).

3.2. Chemical composition of gluten-free rice cookies

The nutrient composition of experimental GF rice cookies (Table 2) appears in line with previous findings (Giuberti et al., 2015b). Differences ($p < 0.05$) among samples were reported for total starch, crude protein and ash contents. In particular, RS_a- and RS_c-cookies had the lowest total starch content (on average 56.4 g/100g DM; $p < 0.05$), whereas an average lower crude protein content was measured for WRS-, RS_a-, RS_b- and RS_c-cookies when compared to CTR-cookies (13.0 *versus* 15.6 g/100g DM, respectively; $p < 0.05$). Differences in the chemical composition have already been obtained in RS-enriched pasta compared to the control (Bustos et al., 2011). In addition, the partial replacement of rice flour with the applied RS ingredients caused a significant rise in the total dietary fibre content, the highest value obtained for RS_a-cookies (15.1 g/100g DM; $p < 0.05$). An enhanced total dietary fibre contents has been reported in wheat pasta and in GF bread samples formulated with different RS sources (Gelencsér et al., 2010; Giuberti et al., 2016). From a nutritional standpoint, to claim that a food is a “source of dietary fibre”, it should contain at least 3 g per

100 g of serving of total dietary fibre, whereas the claim ‘high in dietary fibre” is assigned to food with at least 6 g/100g. Therefore, RS_a- and RS_c-cookies can be considered high dietary fibre food products. Greater amounts of dietary fibre by GF baked products are considered beneficial, since a general low intake of this food component has been described for the coeliac population (Pellegrini and Agostoni, 2015). No differences were reported for crude lipid and free sugar contents, on average being 13.2 g/100g DM and 0.2 g/100g DM, respectively. Average moisture content of 3.2 g/100g cookies was reported, thus indicating a long shelf life of the products (Giuberti et al., 2015b).

3.3. Starch fractions of gluten-free cookies and estimated resistant starch loss of ingredients

As reported in Table 3, values of 31.5, 29.4 and 1.5 g/100g DM were respectively measured for RDS, SDS and RS in CTR-cookies, appearing in line with our previous findings obtained for GF cookies formulated with 100 % GF commercial flour blend (Giuberti et al., 2015b). In addition, compared to CTR-, WRS-cookies were characterized by higher RDS ($p < 0.05$) and a numerically lower RS contents. Data from literature suggest that higher RDS and lower RS contents generally characterized foods containing waxy and/or low-amylose starches when compared to foods formulated with normal amylose starches, being recognized that amylopectin possesses a much larger surface area per molecule than amylose, which makes it a preferable substrate for amylolytic attack (Singh et al., 2010). In all cases, the replacement of a part of commercial rice flour with the three different RS ingredients influenced the starch fraction contents in different ways, indicating that the behaviour of the applied RS ingredients changed during the baking process (Table 3). In particular, RS_a-cookies had the lowest RDS and the highest RS contents (25.6 and 13.3 g/100g DM, respectively; $p < 0.05$), whereas the lowest SDS content was measured in RS_b-cookies (13.0 g/100 g DM; $p < 0.05$). Similar changes have already been reported in GF breads (Giuberti et al., 2016) and in wheat pasta (Bustos et al., 2011; Aravind et al., 2013) formulated with different RS preparations. From a nutritional standpoint, there is general consensus that RDS ingestion promotes a fast increase in blood glucose and insulin levels in human subjects, whereas SDS usually provides a slow and prolonged release of glucose into the blood stream (Raigond et al., 2015). In addition, both RS_a- and RS_c-cookies contained more than 14 % of RS on a total dry starch basis, thus supporting international health claim recommendations (EFSA, 2011).

In the current evaluation, all the three RS preparations have proved effective in increasing the proportion that tested as RS, in line with previous findings (Shi and Gao, 2011; Van Hung et al., 2016a, 2016b). In particular, compared to the RS content of native WRS (13.2 g/100g DM), all obtained RS ingredients had greater RS yield, with values of 71.4 g/100g DM for RS_a (debranched WRS), 65.2 g/100 g DM for RS_b (annealed WRS) and 50.4 g/100g DM for RS_c (acid-heat-moisture treated WRS). Usually, the term heat-moisture treatment is used when low moisture levels (< 35 % w/w) are applied, whereas, annealing refers to treatment of starch in excess (< 65 % w/w) or at intermediate (40-55 % w/w) water levels (Hoover, 2010). As a function of the starting materials and the applied protocols, annealing and heat-moisture treatments can result in structural changes within the amorphous and crystalline regions of starch to different extent, which in turn can influence enzyme susceptibility by either improve the order of the crystalline fraction or enhance the proportion of this fraction (Thompson, 2000). In addition, a limited acid hydrolysis prior to hydrothermal treatments can contribute to the formation of starch resistant to digestion, due to the presence of either short linear chains with enhanced mobility or cross-linking structures between starch chains that appear to participate in the formation of resistant portions through rearrangement and recrystallization of starch during subsequent cooling (Thompson, 2000). Last, since amylopectin chains can interfere with amylose retrogradation, cutting of amylopectin into shorter starch chains with debranching enzyme such as pullulanase can further increase the RS yield (Haralampu, 2000).

The RS content of both raw commercial rice flour and native WRS markedly decreased during the baking process, with estimated RS loss values closer to 90 % (Table 3). Due to the heating of processing, it can be expected that the RS content of raw ingredients will be significantly reduced by disrupting the semicrystalline structure of starch granules during the gelatinization process (Vasanthan and Bhatty, 1998). In addition, RS loss values of 49.5 %, 58.8 % and 90.5 % were estimated for RS_a-, RS_c- and RS_b-ingredients, respectively ($p < 0.05$), thus indicating a different thermal behavior of the applied RS ingredients. Based on these values, we can therefore suppose a heat stability in the order of RS_a (debranched WRS) > RS_c (acid and heat moisture treated WRS) > RS_b (annealed WRS). Despite it has been reported that the annealing treatment of WRS can result in structural changes within the amorphous and crystalline regions that may lead to the formation of a thermo-stable RS complex (Van Hung et al., 2016a), in our experimental conditions RS_b ingredient markedly lost its thermal stability during subsequent baking to an higher extent with respect to RS_a and RS_c ingredients. It is difficult to acquire a consensus on the effect of annealing from literature due to difference in the preparation conditions, starch sources and applied digestion protocols (Hoover, 2010). In addition, high-RS ingredients from WRS have been only analyzed immediately after their preparation, but never after their incorporation into food and the subsequent cooking process. However, Zeng et al. (2015) showed a lesser RS content in WRS subjected to dual hydrothermal treatment (combination of annealing and heat-moisture treatment) when compared to native WRS or to WRS subjected only to annealing treatment. Authors (Zeng et al., 2015) attributed these findings to an increase in starch granule porosity (facilitating enzyme activities) and/or a disruption in those crystallites that were perfect after the single hydrothermal treatment, thus leading to a RS loss during the subsequent heat treatment.

In addition, present findings suggested that treating WRS with pullulanase debranching enzyme prior to the heat treatment may contribute to create more ordered crystalline structures with enough heat stability to maintain their close packing under cooking conditions (Vasanthan and Bhatty, 1998). During debranching, WRS would release relatively short linear fragments similar to amylose that could re-associate leading to a new and strong crystalline structure upon cooking, thereby leading to the formation of a more stable RS complex (Guraya et al., 2001). Likewise, the inclusion of 20 % of RS obtained from debranched HAS from maize contributed to formulate GF-breads with higher RS content more than equivalent amounts of HAS maize subjected to three consecutive autoclaving-cooling cycles, even if RS losses for single ingredients were not reported (Giuberti et al., 2016). In addition, Shi and Gao (2011) reported an increase in the apparent amylose content in the debranched WRS with respect to native WRS. The presence of amylose can affect the RS formation, by reducing the degree of starch swelling during gelatinization and/or by leading to a tightly packed crystalline structure during starch retrogradation on cooling (Haralampu, 2000).

Up to now, no information is present on the RS loss of aforementioned RS ingredients obtained from WRS subjected to a subsequent cooking process after incorporation into cookies. For other food categories and RS sources, contrasting results have been reported. In particular, Gelencsér et al. (2010) found a decreased RS content after cooking (on average -50 %) in RS-enriched wheat pasta samples formulated with RS from HAS of from chemically modified phosphate starch. In contrast, Aravind et al. (2013) did not report changes caused by processing comparing uncooked and cooked pasta samples containing RS from native or from retrograded HAS. Also Aparacio-Saguilán et al. (2007) pointed out to a similar result using

RS from lintnerized banana starch in wheat cookies. Differences in experimental conditions, RS sources and preparations along with different method used for RS determination could explain these discrepancies. Further investigations concerning the relationship between heat stability of various RS formulations and the baking process are therefore required to maximize RS content in the eaten products.

3.4. In vitro glycaemic index of gluten-free cookies

The GI concept has been introduced to classify different carbohydrate-rich foods with respect to their effect on post-meal glycaemia. Accordingly, foods can be classified into three main categories, having low (<55), medium (55–69) and high (>70) GI (Foster-Powell et al., 2002). Nowadays, there is considerable interest in lowering the GI of high digestible foods since a long-term intake of lower-GI foods may favourably influence post-prandial and insulin responses and can be beneficial for prevention and control of obesity and metabolic risk factors (Raigond et al., 2015). Both *in vivo* and *in vitro* methods have been developed to allow the evaluation of GI values and *in vitro* digestion models can represent a viable, rapid and cost effective alternative for the prediction of the *in vivo* GI and for a preliminary screening of new-developed products.

Using WWB as reference, CTR-cookies were characterized by an *in vitro* GI value of 90, in line with previous indications for analogous GF food categories (Foster-Powell et al., 2002) (Table 4). The incorporation of RS ingredients reduced to a different extent the *in vitro* GI of cookies with respect to CTR- and WRS-cookies, the lowest value recorded for RS_a-cookies (i.e., 71; $p < 0.05$). Present findings could be related to the respective RDS and RS contents of individual GF cookie categories, these fractions being respectively related in positive and negative ways to *in vitro* GI values (Giuberti et al., 2012; Aravind et al., 2013). In addition, the increasing amount of total dietary fibre found in RS-enriched GF cookies, along with the possible formation of amylose-lipid complexes during cooking, could have contributed to further reduce the accessibility of amylase to hydrolyse the starch (Singh et al., 2010). Last, different ($p < 0.05$) k values, which reflect the rate of starch hydrolysis, were obtained (Table 4). In particular, RS_a- and RS_c-cookies had the lowest ($p < 0.05$) k values when compared to all other cookies, being 0.017 min^{-1} and 0.022 min^{-1}, respectively. This indicates that starch contained in RS_a- and RS_c-cookies was less susceptible to the digestive enzymes and much slower hydrolysed than starch contained in CTR-, WRS- and RS_b-cookies. The consumption of foods with slowly digestible starch properties may be beneficial for the prevention of hyperglycaemia-related disorders, such as diabetes and cardiovascular diseases (Raigond et al., 2015). However, in order to confirm present *in vitro* evaluations, *in vivo* results are strongly recommended.

3.5. Physical and textural characteristics of gluten free rice cookies

The results of various physical and textural characteristics are shown in Table 5. Significant differences among cookies ($p < 0.05$) were observed in the colour and hardness parameters. In particular, RS_b-cookies displayed the highest L^* and b^* (77.0 and 38.3, respectively; $p < 005$) and the lowest a^* values (1.2; $p < 0.05$). These difference can be related to uneven exposure of cookies' surface area to baking temperature, thus leading to different chemical reactions such us Maillard reactions which occur during baking (Uthumporn et al., 2015). In addition, RS_a-cookies were the hardest in texture, being 67.9 *N* ($p < 0.05$). It is well recognized that hardness of cookies is much affected by the composition of flours and interaction among ingredients. In particular, Norhidayah et al. (2014) reported the highest hardness value for cookies with higher amounts of RS. Presumably, some of the starch granules remained in their native form during baking and did not form a continuous structure, thus leading to an increase in hardness (Norhidayah et al. (2014). In addition, the higher dietary fibre content of RS_a-cookies could have contributed to further increase this value, as already reported in cookies made with eggplant flour (Uthumporn et al., 2015). Last, similar diameter, thickness and spread ratio values were obtained. Cookie spread represents a ratio of diameter and height and, in general, cookies with higher spread ratio are considered the most desirable. Slightly higher, but still comparable, spread ratio values (on average 5.6) have been reported for GF cookies made from flour blends of minor millets (Sharma et al., 2016).

4. Conclusions

Five different GF-cookies were formulated using 100 % rice flour or blends with 50:50 rice flour and native WRS or three different RS ingredients obtained by subjecting WRS to hydrolysis by pullulanase debranching enzyme (RS_a), annealing (RS_b) and a combination of acid and heat-moisture treatments (RS_c). Both thermal and pasting properties differed among starch ingredients. Considering GF-cookies, differences in the chemical composition and in the *in vitro* starch digestion characteristics were reported. In addition, despite all the three RS preparations have proved effective in increasing the total amount of RS, analyses revealed that the heat stability of these RS ingredients decreased in the order of $RS_a > RS_c > RS_b$. Consequently, the higher RS content, along with the lower *in vitro* GI values, were obtained for RS_a-cookies. Among cookies, similar diameter, thickness and spread ratio values were measured, whereas significant differences in colour and hardness were reported. Taking together, present *in vitro* findings suggested that the partial replacement of rice flour with a RS ingredient obtained through debranching WRS could contributed to formulate GF rice cookies with likely slowly digestible starch properties more than equivalent amounts of RS ingredients obtained by subjecting WRS to annealing or acid-heat moisture treatments. Present *in vitro* findings would help to better understand the properties of modified WRS as a potentially source of RS in baked GF products. However, in order to confirm present *in vitro* results, *in vivo* trials are strongly warranted.

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Figure 1. Pasting properties of native (WRS) and debranched, annealed and acid-heat-moisture treated WRS (RS_a, RS_b and $RS_{c,}$ respectively).

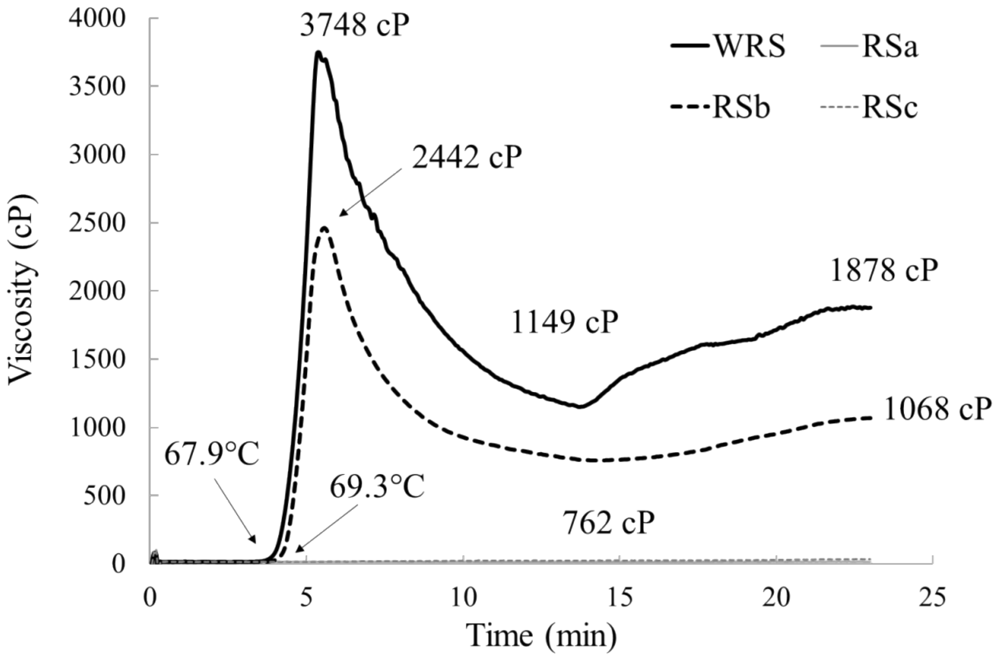
3748 cP
WRS
RSa
RSb
RSc
2442 cP
1878 cP
1149 cP
1068 cP
67.9°C
69.3°C
762 cP
Viscosity (cP)
Time (min)
4000
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25

Figure 1.

Table 1. Thermal properties and water absorption capacity (WAC) of native and treated waxy rice starch samples.

Parameters[1]	Starches			
	WRS	RS_a	RS_b	RS_c
Thermal properties				
T_0 (°C)	67.8±0.12	81.7±0.08	77.4±0.11	79.7±0.12
T_p (°C)	72.4±0.10	93.5±0.13	90.1±0.09	92.1±0.16
T_c (°C)	85.3±0.16	107.2±0.11	95.7±0.11	101.6±0.13
ΔH (J/g)	10.2±0.09	17.8±0.10	12.3±0.09	15.1±0.11
WAC (%)	187±0.1	212±0.2	200±0.1	210±0.2

[1]Experimental data are the means of duplicates±standard deviation.
WRS: native waxy rice starch.
RS_a: RS ingredient obtained from debranched waxy rice starch.
RS_b: RS ingredient obtained from annealed waxy rice starch.
RS_c: RS ingredient obtained from acid and heat-moisture treated waxy rice starch.

Table 2. Chemical composition (g/100g dry matter) of gluten-free rice cookies substituted with different resistant starch (RS) ingredients[1].

Parameters	Experimental cookies					√MSE	p of the model
	CTR	WRS	RS_a	RS_b	RS_c		
Moisture[2]	3.3	3.1	3.4	2.9	3.2	0.46	0.675
Total starch	62.5^b	66.3^c	56.3^a	65.9^c	56.5^a	0.79	< 0.05
Crude protein	15.6^b	13.0^a	13.1^a	13.1^a	12.9^a	0.41	< 0.05
Crude lipid	13.3	13.4	13.0	13.0	13.2	0.40	0.827
Total dietary fibre	3.2^b	2.2^a	15.1^e	5.6^c	10.0^d	0.62	< 0.05
Ash	0.9^a	0.8^a	1.0^a	0.9^a	1.8^b	0.02	< 0.05
Free sugars	0.2	0.2	0.1	0.2	0.2	0.03	0.177

[1]For each recipe, three batches replicates were produced and analyzed in triplicate.
CTR: control gluten-free rice cookies prepared with 100 % commercial rice flour.
WRS: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with native waxy rice starch.
RS_a: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from debranched waxy rice starch.
RS_b: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from annealed waxy rice starch.
RS_c: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from acid and heat-moisture treated waxy rice starch.
[2]g water/100 g food.
Least squares means within lines with different superscripts differed ($p < 0.05$).

Table 3. Starch fraction contents (g/100g dry matter) of gluten-free rice cookies substituted with different resistant starch (RS) ingredients[1] and estimated RS loss (%) of single ingredients.

Parameters	Treatments					√MSE	p of the model
	CTR	WRS	RS_a	RS_b	RS_c		
Gluten-free cookies[2]							
Rapidly digestible starch	31.5^b	46.2^c	25.6^a	49.9^c	30.5^b	1.30	< 0.05
Slowly digestible starch	29.4^c	19.5^b	17.3^b	13.0^a	18.0^b	1.57	< 0.05
Resistant starch	1.5^a	0.6^a	13.3^d	3.0^b	8.0^c	0.47	< 0.05
Ingredients[3]							
Estimated RS loss[4]	86.1^c	96.9^d	49.5^a	90.5^c	58.8^b	3.41	< 0.05

[1]For each recipe, three batches replicates were produced and analyzed in triplicate.
[2]For gluten-free cookies: CTR: control gluten-free rice cookies prepared with 100 % commercial rice flour; WRS: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with native waxy rice starch; RS_a: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from debranched waxy rice starch; RS_b: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from annealed waxy rice starch; RS_c: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from acid and heat-moisture treated waxy rice starch.
[3]For single ingredients: CTR: commercial rice flour; WRS: native waxy rice starch; RS_a: RS ingredient obtained from debranched waxy rice starch; RS_b: RS ingredient obtained from annealed waxy rice starch; RS_c: RS ingredient obtained from acid and heat-moisture treated waxy rice starch.
[4]Estimated on the basis of the expected *versus* the effectively measured RS content of experimental gluten-free cookies after correction for the amount of RS coming from commercial rice flour. The latter was calculated taking into account the RS content of control cookies (100 % rice flour) after the baking process.
Least squares means within lines with different superscripts differed ($p < 0.05$).

Table 4. Starch hydrolysis index (HI), *in vitro* glycaemic index (GI) and rate of starch hydrolysis (k, min^{-1}) of gluten-free rice cookies substituted with different resistant starch (RS) ingredients[1].

Parameters	Experimental cookies					√MSE	p of the model
	CTR	WRS	RS_a	RS_b	RS_c		
HI[2]	95^c	110^d	73^a	100^d	89^b	2.2	< 0.05
in vitro GI	90^c	103^d	71^a	95^c	85^b	2.1	< 0.05
k	0.036^c	0.044^d	0.017^a	0.033^c	0.022^b	0.0018	< 0.05

[1]For each recipe, three batches replicates were produced and analyzed in triplicate.
CTR: control gluten-free rice cookies prepared with 100 % commercial rice flour.
WRS: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with native waxy rice starch.
RS_a: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from debranched waxy rice starch.
RS_b: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from annealed waxy rice starch.
RS_c: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS ingredient derived from acid and heat-moisture treated waxy rice starch.
[2]Calculated using commercial soft white wheat bread as reference (HI = 100)
Least squares means within lines with different superscripts differed ($p < 0.05$).

Table 5. Physical and textural characteristics of gluten-free rice cookies substituted with different resistant starch (RS) preparations.

Parameters	Experimental cookies					√MSE	p of the model
	CTR	WRS	RS_a	RS_b	RS_c		
Diameter (mm)	51.2	51.3	51.6	52.0	50.6	0.68	0.422
Thickness (mm)	10.4	10.4	10.0	10.0	9.8	0.33	0.370
Spread ratio	4.9	4.9	5.2	5.2	5.2	0.18	0.419
L^* (lightness)	66.1^c	69.8^d	64.4^b	77.0^e	62.5^a	0.04	< 0.05
a^* (redness-greenness)	5.7^c	4.5^b	6.8^e	1.2^a	6.4^d	0.03	< 0.05
b^* (yellowness-blueness)	34.8^b	35.8^c	34.8^b	38.3^d	34.1^a	0.02	< 0.05
Hardness (N)	64.3^b	64.9^b	67.9^c	65.6^b	60.0^a	0.47	< 0.05

CTR: control gluten-free rice cookies prepared with 100 % commercial rice flour.
WRS: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with native waxy rice starch.
RS_a: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS derived from debranched waxy rice starch.
RS_b: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS derived from annealed waxy rice starch.
RS_c: gluten-free rice cookie prepared by replacing 50 % of commercial rice flour with RS derived from acid and heat-moisture treated waxy rice starch.
Least squares means within lines with different superscripts differed ($p < 0.05$).