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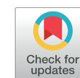
Research article

In vitro evaluation of fermentation characteristics of type 3 resistant starch



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ABSTRACT

Four different resistant starch (RS) type 3 (RS3; retrograded starch) and a RS type 2 (RS2; native high amylose maize starch) were *in vitro* digested and fermented by faecal inoculum. Total and individual short chain fatty acid (SCFA) production and associated kinetic parameters were assessed up to 20 h of *in vitro* fermentation. Total SCFA production was different ($p < 0.05$) among RS-rich ingredients, ranging from 7.43 to 8.72 mmol/g dry starch incubated. Differences ($p < 0.05$) were recorded for propionate and butyrate productions. Different ($p < 0.05$) half-time of total SCFA fermentation ($T_{1/2}$), maximum rate of production (R_{max}) and the time of occurrence (T_{max}) values were measured among RS-rich ingredients, ranging from 3.3 to 5.6 h, from 1.06 to 1.85 mmol/g dry starch incubated per hour and from 2.6 to 4.9 h, respectively. Similar trends were measured considering the fermentative kinetics of individual SCFA. Present preliminary *in vitro* findings indicated that quantitative and qualitative production of SCFA, and inherent fermentation kinetics, were influenced by the type of RS. These findings are based on an *in vitro* approach, thus requiring *in vivo* trials.

1. Introduction

Resistant starch (RS) refers to a portion of dietary starch plus starch degradation products non-absorbed in the small intestine but fermented in the large intestine of healthy individuals (Bird et al., 2013). Several physiological benefits have been ascribed to RS, including, but not limited to, a positive impact on blood glucose and lipid profiles, along with a possible role on bowel health maintenance (Wong et al., 2006). As dietary fibre, several beneficial effects associated to the RS consumption are mediated through its fermentation by the gut microbiota. In particular, the main end-products of RS fermentation are gases and short chain fatty acids (SCFA), mainly acetate, propionate and butyrate. The primary effects of SCFAs are on colonic functions, although they can also act as metabolic substrates for other tissues (Wong et al., 2006; Bird et al., 2013). When compared to the fermentation behaviour of other types of dietary fibre, a range of *in vivo* and *in vitro* studies revealed that RS may act as a fermentative substrate that stimulates butyrate production, whereas much lesser amounts of organic acids (*e.g.,* lactate and succinate) are produced (Bird et al., 2007, 2013).

There is growing interest on non-digestible but fermentable food components that may benefit the host by increasing the amount of SCFA and/or by selective stimulating the growth or activity of beneficial bacteria in the colon, including different types of RS derived from various sources. Nowadays, *in vitro* fermentation models using human or pig faeces *inoculum* are commonly used as alternatives to *in vivo* trials to estimate SCFA production, especially for screening of novel substrates (Roura et al., 2016). Accordingly, most scientific research has been conducted to investigate the *in vitro* fermentation of various types of RS, with the greatest attention on RS type 2 (RS2) from native high amylose maize starch (Bird et al., 2007). On the contrary, relatively little information is present on the fermentation behaviour of other types of RS, including RS type 3 (retrograded starch as a result of processing; RS3) (Wandee et al., 2017). This may be concerning since RS3-rich ingredients can promote a greater preservation of RS in cooked food products than the RS2 counterparts, due to a greater thermal stability in most cooking operations (Zhang et al., 2012; Wandee et al., 2017; Giuberti et al., 2019).

This paper aimed to compare the total and individual SCFA profiles and related kinetics of four different RS3-rich ingredients using an *in vitro* approach based on enzymatic digestion followed by *in vitro* large intestine fermentation. For comparison purpose, native high amylose maize starch (*i.e.,* RS2) was included, being one of the most common commercial type of RS used in food product formulation.

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2. Materials and methods

2.1. Commercial resistant starch rich ingredients

Three different commercially available RS-rich ingredients were chosen (Table 1), including retrograded high-amylose maize starch (RS3m; Novelose 330; Ingredion Incorporated, Westchester, USA), retrograded tapioca starch (RS3t; C☆Actistar™ 11700; Cargill, Wayzata, USA) and native high-amylose maize starch (RS2; S4180, Sigma-Aldrich Co. Milan, Italy), the latter used for comparison purposes.

2.2. Novel resistant starch rich ingredients

Two novel RS-rich ingredients were also included (Table 1). In particular, novel retrograded sorghum starch (RS3s) was obtained by applying the procedure of annealing to isolated white sorghum starch as detailed by Giuberti et al. (2019). After dispersion of isolated sorghum starch in distilled water (1:4 w/v starch to water), the mixture was incubated at 50 °C for 24 h under continuous stirring. The excess of water was removed by centrifugation and the resulting pellet containing retrograded white sorghum starch was oven dried at 40 °C.

Novel retrograded waxy rice starch (RS3r) was produced by debranching gelatinized rice starch (95 °C for 30 min) by pullulanase enzyme (10% w/w starch in a pH 4.5 buffer solution, 55 ASPU/g dry starch of heat stable pullulanase; 58 °C; 12 h) (Giuberti et al., 2017).

2.3. Resistant starch quantification

The RS content of each RS-rich ingredient was measured in triplicate with an enzymatic assay kit (Megazyme assay kit K-RSTAR 05/19) (Table 1). Briefly, samples were incubated at 37 °C with a buffered solution containing pancreatic α-amylase (3 Ceralpha U/mg; 10 mg/mL) and amyloglucosidase (3,300 U/mL on soluble starch) enzymes. After 16 h of incubation, ethanol was added, samples were centrifuged and the resulting pellet containing the RS fraction was purified with ethanol and solubilized with 2 M KOH. Then, 1.2 M sodium acetate buffer was added, and starch was hydrolyzed to D-glucose with amyloglucosidase (0.1 mL) at 50 °C for 30 min. The amount of RS was measured at 510 nm.

2.4. In vitro digestion and large intestine fermentation

Each native RS-rich ingredient was subjected to an *in vitro* digestion step (*i.e.,* oral, gastric and pancreatic phases) prior to *in vitro* fermentation as detailed by Minekus et al. (2014) with minor modifications. Briefly, the digestion process (scaled up for 10 g of sample) included: *i*) an oral phase (composed by a simulated salivary fluid at pH = 7.0 containing human salivary alpha-amylase, 75 U/ml; A0521; Sigma-Aldrich Co. Milan, Italy) at 37 °C for 2 min; *ii*) a gastric phase (composed by a simulated gastric fluid at pH 3.0 containing pepsin, 2000 U/ml; P7000; Sigma-Aldrich Co. Milan, Italy) at 37 °C for 120 min; *iii*) an intestinal phase composed by a simulated intestinal fluid (pH = 7.0) containing pancreatin at 100 U/ml (P1750; Sigma-Aldrich Co. Milan, Italy) and bile salts, 10 mM (B8631; Sigma-Aldrich Co. Milan, Italy) at 37 °C for 120 min. The digestion was stopped by cooling on ice. Then, undigested residues were recovered, washed with ethanol (2 × 25 ml; 95% ethanol) and air-dried overnight. Duplicate dried samples were pooled and ground to pass through a 60-mesh screen.

The *in vitro* large intestine fermentation was conducted as reported by Jonathan et al. (2012) with minor modifications. Fresh faeces were collected from five growing pigs (Italian Large White × Italian Duroc; 38.4 ± 3.65 kg body weight; 3–4 month of age) from the production herd of the Research Centre for Zootechny and the Environment (CERZOO S.r.L; S. Bonico, Piacenza, Italy). Pigs had free access to water and were fed a commercial diet devoid of antibiotics formulated according to meet nutrient requirements (NRC, 1998). Freshly voided faecal samples were captured immediately after physiological defecation, placed in airtight plastic syringes, kept at 39 °C and processed within 20 min after collection. Animal care and use practices during this trial conformed to the guidelines of the European Parliament and Council (2010/63/EU Directive).

Dried pooled post-digestion residues were weighed (*i.e,* 400 mg) in triplicate into 125-ml amber glass bottle filled with 60 ml of a filtered buffer solution prepared according to Williams et al. (2005). The CO_2-saturated fermentation medium contained 0.05 g/ml of fresh faeces obtained by pooling equal amounts (wet weight) of faeces from each animal. Bottles were sealed with a rubber stopper and placed in a shaking water bath (60-rpm) at 37 °C. Three bottles without substrate were used as control of background fermentation. Three incubation runs were conducted in 3 different days, considering bottles within runs as repetitions and bottles between runs as replicates. Aliquots (2.0 ml) were aseptically removed from each bottle at 4, 8, 16, and 20 h of incubation and stored at -20 °C.

Table 1. Resistant starch ingredients subjected to an *in vitro* digestion and large intestine fermentation system.

	Characteristic	RS (g/100 g DM)
Resistant starch		
RS3m[1]	Retrograded high-amylose maize starch	46.9 ± 3.21
RS3t[2]	Retrograded tapioca starch	47.5 ± 2.42
RS3s[3]	Retrograded sorghum starch	52.3 ± 2.17
RS3r[4]	Retrograded waxy rice starch	54.6 ± 2.02
RS2[5]	Native high amylose maize starch	55.1 ± 2.72

Abbreviations: RS: resistant starch; DM: dry matter.
[1] Novelose 330 (Ingredion Incorporated).
[2] C☆Actistar™ 11700 (Cargill).
[3] Annealed white sorghum starch.
[4] Debranched waxy rice starch.
[5] S4180 (Sigma-Aldrich Co.).

2.5. Short chain fatty acid analysis and calculations

Aliquots were analyzed for the SCFA content by gas chromatography (Varian 3350 system, Varian Inc., CA). The apparatus consists of a silica capillary column (DB-5, Agilent Technologies, USA) and pivalic acid was used as internal standard. The individual SCFA content (*i.e.,* acetate, propionate and butyrate) was blank-corrected and the total SCFA production ($SCFA_{tot}$, mmol/g dry starch incubated) was calculated as the sum of the individual SCFA contribution. Profiles of SCFA production were fitted to a monophasic model (Eq. 1) (Groot et al., 1996):

$$G = A/(1+(Ct))^{B} \tag{1}$$

where G is the total or individual SCFA production, A is the asymptotic production, B is the switching characteristic of the curve, C is the time at which half of the asymptote has been reached ($T_{1/2}$) and t is the time (h). The maximum rate of total and individual SCFA production (R_{max}) and the time at which it occurs (T_{max}) were calculated (Eq. 2 and Eq. 3, respectively) (Bauer et al., 2001):

$$R_{max} = (A(C^{B})B(T_{max}^{(-B-1)}))/(1+(C^{B})(T_{max}^{(-B)}))^{2} \tag{2}$$

$$T_{max} = C(((B\text{-}1)/(B+1))^{(1/B)}) \tag{3}$$

2.6. Statistical analysis

Data were tested by Kolmogorov-Smirnov test and Shapiro-Wilk test for normal distribution. Data were subjected to ANOVA using the GLM procedure of SAS (2003) and main effect into the model was the RS-rich ingredient. Significance was declared at $p < 0.05$.

Table 2. Total and individual production of short-chain fatty acids (mmol/g dry starch incubated) after 20 h of fermentation of different types of resistant starch (n replicates = 3).

	$SCFA_{tot}$	Acetate	Propionate	Butyrate
Resistant starch[1]				
RS3m[2]	7.73a	3.77a	1.87a	1.93b
RS3t[3]	7.43a	3.71a	2.09a	1.45a
RS3s[4]	8.72b	3.93a	2.36b	2.18b
RS3r[5]	7.45a	3.63a	1.96a	1.70a
RS2[6]	7.61a	3.92a	2.04a	1.50a
$\sqrt{MSE}$	0.414	0.257	0.160	0.146

Within each column, means with different letters differed at $p < 0.05$. Abbreviations: $SCFA_{tot}$: total production of short chain fatty acids; RS: resistant starch.

[1] The resistant starch fractions were obtained by subjecting each ingredient to an *in vitro* digestion step composed by oral, gastric and pancreatic phases.

[2] Novelose 330 (Ingredion Incorporated).

[3] C☆Actistar™11700 (Cargill).

[4] Annealed white sorghum starch.

[5] Debranched waxy rice starch.

[6] Native high amylose maize starch (Sigma-Aldrich Co.).

3. Results and discussion

3.1. Total short chain fatty acid production

The interest in thermostable forms of RS for possible food application is rising. In particular, thermally stable RS-rich ingredients can be added to foods with the aim to compensate RS losses derived from food processing (Wandee et al., 2017; Giuberti et al., 2019). To date, other than commercial RS3 ingredients (i.e., RS3m and RS3t), novel types of RS have been characterized for potential food application. These may include, but are not limited to, RS obtained from retrograded nongranular starch (i.e., RS3) and type 4 RS (i.e., RS4), obtained through a chemical process (e.g., etherization, esterization, cross-bonding) that can render selected starches more resistant to the enzyme hydrolysis (Homayouni et al., 2014). In this context, RS3s derived from isolated white sorghum starch subjected to annealing, whereas RS3r was obtained from native waxy rice starch after debranching by the action of pullulanase enzyme. These methods have been successfully applied to native starch (i.e., source of RS2) with the aim to promote the formation of heat-stable forms of RS by altering to different extent the internal rearrangement of starch. In particular, the annealing method can promote structural changes within the amouphous and crystalline regions of starch, whereas the debranching procedure can allow obtaining greater number of linear short chains from amylopectin, thus contributing to increase the yield of retrograded starch (Giuberti et al., 2017, 2019). These novel RS-rich ingredients have been deeply investigated in terms of functional and physicochemical properties, showing greater thermal stability (measured by differential scanning calorimetry, DSC), differences in the pasting properties (measured by Rapid Visco-Analyzer) as well as in the ratio of ordered starch to amorphous starch (measured by FT-IR analysis) when compared to the RS2 counterparts (Giuberti et al., 2017). However, information regarding their fermentation behavior is still scarce. A better understand of the prebiotic role of different types of native and modified RSs is crucial, in an effort to better explore potential health benefits mainly related to their unique fermentation behavior in terms of total and individual SCFA productions and related kinetic parameters (Ma and Boye, 2018).

Different total and individual SCFA productions were measured among the selected RS types ($p < 0.05$; Table 2). In particular, the greatest $SCFA_{tot}$ production was obtained for RS3s (*i.e.,* 8.72 mmol/g dry starch incubated, $p < 0.05$). Acetate was produced to the greatest extent (about 50 % of the $SCFA_{tot}$ production) and without difference ($p > 0.05$) among RS-rich ingredients. The greatest propionate level was obtained after the *in vitro* fermentation of RS3s (*i.e.,* 2.36 mmol/g dry starch incubated, $p < 0.05$). Comparable values have been reported through *in vitro* fermentation of different RS-rich ingredients (mainly RS2) (Giuberti et al., 2013). In addition, by using an *in vitro* fermentation system with human faeces as inoculum, Jonathan et al. (2012) reported a total SCFA production of 7.6 and 7.7 mmol/g organic matter for retrograded tapioca starch and retrograded maize starch (*i.e.,* RS3), respectively.

As reviewed by Ma and Boye (2018), chemical and structural characteristics of RS can contribute to affect both total and individual SCFA productions. In particular, Zhou et al. (2013) postulated that the molecular structure of RS is one of the main factors affecting SCFA production in amount and proportion. Authors indicated that RS-rich ingredients with different levels of organization of the starch structure (changes in the FT-IR ratio of absorbance at 1047 and 1022 cm^{-1}) influenced total and individual SCFA formation in an *in vitro* fermentation system. In addition, findings of Dongowski et al. (2005) supported the hypothesis that the fermentation properties of RS raised with increasing heat-stability and crystallinity of the selected RS-rich ingredients. Accordingly, RS3s has been previously characterized by enhanced crystallinity at the surface of the starch granules (measured by FT-IR) and by increased transition temperatures on the DCS thermograms and thermal stability (*i.e.,* onset and peak temperatures >78 °C and transitional enthalpy changes >14.0 j/g) as a result of annealing (Giuberti et al., 2019).

3.2. Butyrate production

As reviewed by Brouns et al. (2002), butyrate is the principal oxidative fuel of colonocytes and induces a number of physiological effects on cell metabolism, maintenance of the epithelial barrier and improvement of the immune defense. In addition, *in vivo* studies conducted in rodents highlighted the potential role of butyrate in alleviating diet-induced obesity and insulin resistance (Liu et al., 2018). Lastly, it has been hypothesized that the ingestion of indigestible carbohydrates as an indirect source of butyrate can be beneficial for reducing the risk factors for colorectal cancer (Wong et al., 2006). These facts prompted us to focus on butyrate. The greatest butyrate production was measured for RS3m and RS3s (*i.e.,* 1.93 and 2.18 mmol/g dry starch incubated, $p < 0.05$; Table 2) and did not differ among the other RS-rich ingredients. Present *in vitro* results are supported by the *in vivo* findings of Venkataraman et al. (2016), where diets rich in RS increased human faecal butyrate concentrations from 8 to 12 mmol/kg wet faeces. Despite it has been reported that RS3 fermentation can result in relatively greater butyrate production with respect to RS2 (Brouns et al., 2002; Lehmann et al., 2002), our *in vitro* data indicated that the butyrogenic properties were markedly affected by the type of RS. In particular, different RS-rich ingredients might vary in their fermentation profile as a function of the nature of the starch, the treatment applied to retrograded starch, as well as the physicochemical and structural properties of the resulting RS-rich ingredients (Jacobasch et al., 2006; Wandee et al., 2017). Accordingly, the butyrogenic response to RS was related not only to the quantity of RS reaching the large intestine, but also to the type of RS (Ma and Boye, 2018; Baxter et al., 2019). Structural changes unique to each RS-ingredient could have influenced the SCFA production. For instance, Lehmann et al. (2002) reported that RS3-rich ingredients from banana starch with distinctive degree of crystallinity and crystalline polymorphs induced different butyrate productions in a human gut *in vitro* fermentation model. In addition, structural changes occurring to RS-ingredients during the *in vitro* fermentation process, as well as the extent at which the substrate was available to microbiota, could contribute to selectively increase the proliferation of butyrate-producing bacteria (Tiwari et al., 2019).

Table 3. Fitted kinetic parameters of total short chain fatty acid ($SCFA_{tot}$), acetate, propionate and butyrate productions following fermentation of different resistant starch rich ingredients (n replicates = 3).

	In vitro fermentation kinetics[1]											
	$SCFA_{tot}$			Acetate			Propionate			Butyrate		
	$T_{1/2}$	R_{max}	T_{max}	$T_{1/2}$	R_{max}	T_{max}	$T_{1/2}$	R_{max}	T_{max}	$T_{1/2}$	R_{max}	T_{max}
Resistant starch												
RS3m[2]	4.5b	1.85b	4.0b	4.5b	0.98c	4.1c	4.6b	0.42b	4.0c	4.6a	0.42b	4.0b
RS3t[3]	3.3a	1.76b	2.6a	3.5a	0.79b	2.6a	3.0a	0.52b	2.4a	3.6a	0.38b	2.9a
RS3s[4]	5.6c	1.54a	4.9c	5.7b	0.76b	5.0d	6.0c	0.34a	5.0d	6.2b	0.43b	4.7c
RS3r[5]	4.7b	1.14a	3.1a	5.1b	0.63a	3.3b	4.4b	0.33a	3.0b	5.2a	0.20a	3.0a
RS2[6]	5.6c	1.06a	4.2b	5.5b	0.64a	4.3c	5.6c	0.25a	3.9c	6.3b	0.19a	4.9c
$\sqrt{MSE}$	0.48	0.278	0.16	0.88	0.168	0.16	0.47	0.074	0.24	1.27	0.082	0.32

Within each column, means with different letters differed at $p < 0.05$. Abbreviations: RS: resistant starch.

[1] $T_{1/2}$: time to reach half of the maximum production (h); R_{max}: maximum rate of production (mmol/g dry starch incubated per hour); T_{max}: time of occurrence of R_{max} (h).

[2] Novelose 330 (Ingredion Incorporated).

[3] C☆Actistar™ 11700 (Cargill).

[4] Annealed white sorghum starch.

[5] Debranched waxy rice starch.

[6] Native high amylose maize starch (Sigma-Aldrich Co.).

3.3. Fermentation kinetic parameters

The monophasic model well fitted the observed values (data not shown). The fermentation kinetic parameters for total and individual SCFA production differed among RS-rich ingredients ($p < 0.05$; Table 3). In particular, $T_{1/2}$, R_{max} and T_{max} values for $SCFA_{tot}$ ranged from 3.3 to 5.6 h, from 1.06 to 1.85 mmol/g dry starch incubated per hour and from 2.6 to 4.9 h, respectively ($p < 0.05$). Similar kinetic trends were measured considering the production of acetate, propionate and butyrate. These differences may be related to the rate of substrate depolymerization by bacterial hydrolytic enzymes prior to the fermentation process (MacFarlane and MacFarlane, 1993). In addition, by focusing on butyrate production kinetics, RS3s and RS2 were characterized by the greatest $T_{1/2}$ value (*i.e.,* 6.2 and 6.3 h, $p < 0.05$). It has been suggested that the time required to reach half of the maximum production (*i.e.,* $T_{1/2}$) can be used as indicator of the probable site of fermentation (Tiwari et al., 2019). Lower $T_{1/2}$ values might denote faster fermentation starting from the proximal segments, whereas higher $T_{1/2}$ values mean a slower SCFA production more located in the distal segments (Williams et al., 2005). Differences in local SCFA production, especially butyrate, may play a role in the maintenance of the colonic health, with implication on the physiological effectiveness of each RS product. Being the distal part of the human large intestine the site most at risk of pathologies, fermentable ingredients that specifically increase SCFA availability in the distal colon can have a positive role in the prevention of certain diseases (Wong et al., 2006).

4. Conclusions

This *in vitro* work was a preliminary study to explore the pattern of short chain fatty acid production of various resistant starch-rich ingredients (type 2 and type 3) fermented through an *in vitro* model. These *in vitro* findings revealed that quantitative and qualitative production of short chain fatty acids and related kinetics were influenced by the type of resistant starch. The greatest $SCFA_{tot}$ production (*i.e.,* 8.72 mmol/g dry starch incubated; $p < 0.05$) was obtained following fermentation of RS3s (annealed white sorghum starch), with no difference among the other RS-rich ingredients. Greatest butyrate productions were measured for RS3m (retrograded high-amylose maize starch) and RS3s (annealed white sorghum starch), being on average 2.05 mmol/g dry starch incubated ($p < 0.05$) and did not differ among the other RS-rich ingredients. Fermentation kinetic parameters for total and individual SCFA production differed among RS-rich ingredients. Focusing on the butyrate production kinetics, RS3s and RS2 (native high amylose maize starch) were characterized by the greatest $T_{1/2}$ value (*i.e.,* 6.2 and 6.3 h, respectively; $p < 0.05$), thus hypothetically indicating a favorably butyrate production more located in the distal segments of the large intestine. It must be pointed out that present findings are based on an *in vitro* approach and thus require future *in vivo* experiments in humans to confirm these results.

Declarations

Author contribution statement

G. Giuberti: Conceived, designed and perform the experiments; Analyzed and interpreted data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A. Gallo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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