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On the Effect of Lactic Acid on Blood Glucose and Insulin Responses to Cereal Products: Mechanistic Studies in Healthy Subjects and *In Vitro*

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ABSTRACT

It has been observed that bread containing lactic acid produced during the sourdough fermentation or added directly, has the ability to lower the postprandial glucose and insulin responses in humans. The main objective of the present work was to evaluate the possible mechanisms for a lowered glucose response to bread containing lactic acid, and to determine whether the same phenomenon also occurs when lactic acid is added to other cereal products. The rate of starch hydrolysis in bread and bread-like products was studied using an *in vitro* enzymatic approach. In addition, blood glucose and insulin responses to different lactic acid fermented barley gruels were evaluated in healthy subjects. It was concluded that the inclusion of lactic acid in bread reduces the rate of starch digestion by creating interactions between the gluten and starch. The presence of lactic acid during starch gelatinisation appeared to be a prerequisite for a reduced starch bioavailability. No effect of lactic acid was seen in gruels where the acid was formed after heat-treatment.

Keywords: lactic acid, bread, glucose response, starch hydrolysis.

INTRODUCTION

Since the early 1980s, the glycaemic index (GI) has been used as a tool for classifying carbohydrate foods according to their effects on postprandial glycaemia[1]. The GI is defined as the area under the blood glucose curve following ingestion of a test food, expressed as a percentage of the corresponding area following an equivalent load of a reference carbohydrate[1]. In the latest report from a joint FAO/WHO expert consultation on carbohydrates[2], an increased intake of low-GI foods was strongly recommended. The rationale for these recommendations are studies demonstrating a therapeutic effect of such foods in patients with diabetes and dyslipidaemia[3] and, in addition, their preventive potential against type II diabetes[4,5] and myocardial infarction[6].

A number of food factors appear to affect the rate of glucose delivery to the blood. Some are related to the characteristics of the raw food, whereas others are related to the processing conditions. The monomeric composition of the carbohydrate moiety may play a role, and the GI of pure, low-molecular weight carbohydrates decrease in the following order; glucose > sucrose > lactose > fructose. In the case of starch, the crystallinity of the substrate is

ABBREVIATIONS USED: DNS = 3,5-dinitrosalicylic acid; GI = glycaemic index; II = insulinaemic index; HI = hydrolysis index; Lac = lactic acid.

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important, and the highly ordered structure present in the native granule constitutes a barrier to enzymatic attack[7]. The starch crystallinity induced upon heat treatment and cooling (retrogradation), may also create regions of highly ordered structures, which are less readily hydrolysed by amylases, or in some cases even render the starch totally resistant, so called resistant starch[7]. Various ways of processing can also create a highly ordered physical food form that renders the starch less readily available for digestion. For example, the macromolecular interactions in pasta, that are responsible for the pasta texture, are also responsible for the low GI properties of this food group[8]. The presence of certain food components may also affect glycaemia, and it has been known for long that viscous, soluble dietary fibre has the capacity to lower the rate of glucose delivery to the blood by lowering the rate of gastric emptying, and/or increasing the thickness of the unstirred layer of the small intestinal mucosa, thus creating an absorption barrier[9]. Organic acids are other components that may be present in the raw foods, produced upon fermentation processing or added, as in the case of pickled food.

It is known that certain acids, such as acetic, propionic, and lactic acid have the ability to lower the postprandial blood glucose and insulin responses, when included in bread meals. In the case of acetic acid added to bread meals, both Liljeberg *et al.*[10] and Brighenti *et al.*[11] have shown blood glucose lowering effects. In the study by Liljeberg *et al.*[10], a lowered gastric emptying rate was detected[10], whereas no such effect was seen by Brighenti *et al.*[11].

Studies addressing the possible mechanisms whereby acids may influence the rate of postprandial glucose delivery to the blood are scarce. However, by use of either direct (ultrasonography) or indirect (paracetamol marker) measurement of the rate of gastric emptying, it was concluded that the lowering of postprandial glycaemia to bread with added sodium propionate in healthy humans, was due to a reduced gastric emptying rate[12,13]. Todesco *et al.*[14], on the other hand, suggested a mechanism related to a reduced rate of starch digestion when studying a similar propionate-enriched bread[14]. In contrast to this finding, Liljeberg *et al.*[15] was not able to show any inhibition of *alpha*-amylase by sodium propionate, when using a buffered enzymatic *in vitro* system. The presence of lactic acid in bread or in mixed meals with vegetables has also been reported to reduce acute glycaemic and/or insulinaemic responses[15–17]. However, the lowering of glycaemia and insulinaemia to bread with added lactic acid could not be attributed to a reduced gastric emptying rate[12]. The glycaemia to such a bread product could instead be predicted from the rate of *in vitro* starch digestion, suggesting a mechanism related to a slowing of the digestive phase[15].

Thus, it appears that at the gastro-intestinal level the mechanism may differ for different acids, and although *in vitro* data are at hand in support of a digestion barrier in the case of bread with added lactic acid, the specific mechanism at the food level has not been identified.

The purpose of the present study was to evaluate further the mechanisms responsible for the lowered availability of starch for amylolysis in bread with lactic acid, using an *in vitro* enzymatic approach. Secondly, the impact of the lactic acid on the rate of *in vitro* starch hydrolysis and metabolic responses in healthy humans were also investigated using a lactic acid fermented barley gruel, where the lactic acid was generated after, instead of prior to, heat treatment. In the case of the gruel product, two different starter cultures were used, one producing only the L-form of lactic acid, and one producing both the L- and D-forms.

EXPERIMENTAL

Amylase kinetics; influence of lactic acid

The kinetics of pancreatic *alpha*-amylase was studied at pH 6·9. Soluble starch (acc. to Zulkowsky GR[18]; MERCK 101257, Darmstadt, Germany) was used as a substrate for the *alpha*-amylase. The reaction rate ν (mg/mL s) was measured, in duplicates, at three different substrate concentrations [S]; 47·5, 23·8, and 11·9 mg/mL available starch. The amount of *alpha*-amylase used was 86 Sigma Units per 1 g available starch and the corresponding amount of lactic acid was 0·31 mmol. Tannic acid, a known inhibitor of *alpha*-amylase[19] was used at a concentration of 0·94 mg/mL. The enzyme solutions, with and without lactic acid or tannic acid, were pre-incubated at 37 °C for 30 min. Enzyme solution, 1 mL, was added to 10 mL of substrate at 37 °C. Duplicate samples were taken after 150, 180, and 210 s, followed by immediate mixing with 0·8 mL phosphate buffer (0·05 M, pH = 6·9) and 1 mL DNS (3,5-dinitro-salicylic acid). All samples and a series of maltose standards were held in boiling water for 10 min and after cooling, 13 mL of distilled water was added. The final absorbance was read at 530 nm.

In vitro starch hydrolysis

In the analysis of the rate of starch hydrolysis the procedure simulates the *in vivo* digestion of starch[20]. Starch is hydrolysed by *alpha*-amylase and the liberated maltodextrins was determined spectrophotometrically by the absorption at 530 nm after reaction with the DNS reagent[21]. The hydrolysis index (HI) for a test product is calculated from the area under the hydrolysis curve divided by the corresponding area for a reference product.

Simulated baking of starch/gluten mixtures with and without added lactic acid

A simplified model system containing starch and gluten was studied. Wheat starch (Cerestar, Germany), corresponding to 1 g available starch, was mixed with gluten (15% on starch basis) and 10 mL phosphate buffer (0·05 M, pH = 6·9). The pH was adjusted, with either lactic or hydrochloric acid, to 4·0, which corresponded with the pH in a previously studied sourdough bread[15]. When lactic or hydrochloric acid was added before starch gelatinisation, the acid was first added to the buffer, the pH adjusted and finally the acid containing buffer was mixed with the starch and gluten. The starch/gluten mixture was incubated to mimic a baking procedure, i.e. 22 °C for 50 min, then 38 °C for 20 min followed by 100 °C for 15 min. In one experiment the starch/gluten mixture was homogenised after the incubation and then 10 g sample was homogenised for 60 s (Polytron PT10/35). In another experiment, lactic acid was added to the starch/gluten mixture immediately after gelatinisation. After cooling to ambient temperature the HI was determined (as described above) for portions of all samples. Wheat starch suspended in water was used as the reference.

Bread with lactic acid; impact of homogenisation

The reference white wheat bread was baked according to Liljeberg and Björck[22] and a similar bread was baked with addition of lactic acid to the dough (2 g/100 g water). The baking was performed in a home baking machine (El-Gennel HB-021). Prior to homogenisation, 35 g bread was mixed with 45 g water for 30 s in a mixer (Philips HR1392). The mixture was then transferred to a glass tube and homogenised for 60 s (Polytron PT10/35). HI was determined for both intact and homogenised lactic acid bread, using intact white bread as the reference.

Pilot-plant baking of lactic acid bread; influence of protein content of flour and addition of gluten

The reference white wheat bread and a series of three lactic acid breads were baked in a pilot-plant bakery (Nord Mills AB, Malmö, Sweden). The ingredients of the breads are listed in Table I. The breads differed from each other in the protein content of the flour and in one case by the addition of gluten. HI was determined for all three lactic acid breads, using the wheat bread without lactic acid as the reference.

Meal studies in healthy humans with fermented gruels containing L- or L/D-lactic acid

Gruel recipes

Three different gruels were prepared; one with L-lactic acid, one with L-/D-lactic acid, and one that was not fermented. The gruel was prepared by mixing 1 L tap water with 185 g high amylose barley flour (hull-less Glacier, 42% amylose, Swalöf-Weibull, Svalöv, Sweden), 9·25 g malt flour and 5 mL of an amylolytic enzyme preparation (*SAN Super*, Novo Nordisk A/S, Copenhagen, Denmark). The mixture was heated to 55 °C and held at this temperature for 1 h, then heated to 95 °C. On reaching 95 °C, the mixture was immediately allowed to cool to an ambient temperature. The gruel was then fermented by adding 1·2 mL ($1 \cdot 10^7$ CFU/mL) of a homofermentative starter culture and incubated in 37 °C overnight (16 h). The L-/D-lactic acid gruel was fermented with

Table I List of ingredients and final protein content in pilot-plant bread products

	Wheat bread	Wheat bread + lactic acid		
Ingredient	Flour no. 1 (g)	Flour no. 1 (g)	Flour no. 2 (g)	Flour no. 2 + Gluten
Wheat flour	1620	1710	1710	1675
Water	1000	980	980	980
Yeast	46	46	46	46
Monoglycerides	33	33	33	33
Sucrose	20	20	20	20
Salt	13	13	13	13
Lactic acid	—	20	20	20
Gluten	—	—	—	20
Protein % wet wt.	6·5	6·2	7·3	7·8

a *Lactobacillus plantarum 299* and *Lactobacillus plantarum 16M2* starter culture. The L-lactic acid gruel was fermented with *Lactobacillus rhamnosus 271* only. The pH in both fermented gruels, when served as breakfast meals, was 3·4–3·5.

Study design

Ten healthy non-smoking volunteers, eight women and two men, aged 23–53 year, with normal body mass indices ($21{\cdot}5 \pm 0{\cdot}5\,kg/m^2$) and without drug therapy participated in the study. The meals contained 270 g of gruel or 59 g of white bread. Water (250 mL) was served with the white bread reference meal and tea or coffee (150 mL) was served with both reference and test meals. Butter (80% fat) and cheese (10% fat) were used to balance the fat and protein contents of the meals. All meals contained 25 g available carbohydrates, 5·3 g protein and 1·7 g fat, providing 580 kJ (138 kcal). The subjects were served the test meals in a random order on four separate occasions, at the same time in the morning, following an over-night fast. All meals were consumed steadily over a 12–14 min period. Finger-prick capillary blood samples were taken prior to the meal (0 min) and at 15, 30, 45, 70, 95, 120, and 180 min after the meal for the analysis of glucose, and at 0, 15, 30, 45, 95, and 120 min for the analysis of insulin. Blood glucose concentration was determined with a glucose oxidase peroxidase reagent and serum insulin level with an enzyme immunoassay kit (Boehringer Mannheim, Germany). The study was performed during a period of three months and all subjects were aware that they could withdraw from the study at any time they desired. The study was approved by the Ethics Committee of the Faculty of Medicine at Lund University.

Statistical methods

In calculating HI, GI and II, the areas under the curves (AUC) were used (GraphPad Prism, ver. 3·0; Graph Pad Software, San Diego). Each subject provided their own reference and all areas below the baseline were excluded from the calculations. Values are presented as mean ± SEM. All statistical calculations were performed in MINTAB Statistical Software (release 13 for Windows; Minitab Inc, State College, PA). Significances were evaluated with the general linear model (analysis of variance), followed by Tukeys multiple comparisons test. Values of $P < 0{\cdot}05$ were considered significant.

RESULTS

Amylase kinetics; influence of lactic acid

A Lineweaver-Burk plot at pH 6·9 did not reveal any inhibitory effect of lactic acid, when the lactic acid was pre-incubated with *alpha*-amylase (Fig. 1). In contrast, tannic acid, a known *alpha*-amylase inhibitor, yielded a V_{max} of $-2 \cdot 10^{-3}$ as compared with a V_{max} of 0·029 for the reference.

In vitro starch hydrolysis

Simulated baking of starch/gluten mixtures with and without added lactic acid

The hydrolysis indices (HI) for the starch/gluten mixtures are shown in Figure 2. The HI of a starch/gluten mixture containing lactic acid (SGL) was 22% lower ($P < 0{\cdot}05$) than the corresponding mixture without the acid (SG). When gluten was absent (SL) or when lactic acid was replaced for HCl (SGH) to a corresponding pH (4·0), no lowering of HI was seen. Also, when the SGL mixture was homogenised after gelatinisation (SGLhom) the HI was 25% higher, than for the non-homogenised SGL ($P < 0{\cdot}05$). When lactic acid was added after the gelatinisation (SGLafter), the HI was not lowered to the same extent as for SGL ($P < 0{\cdot}05$).

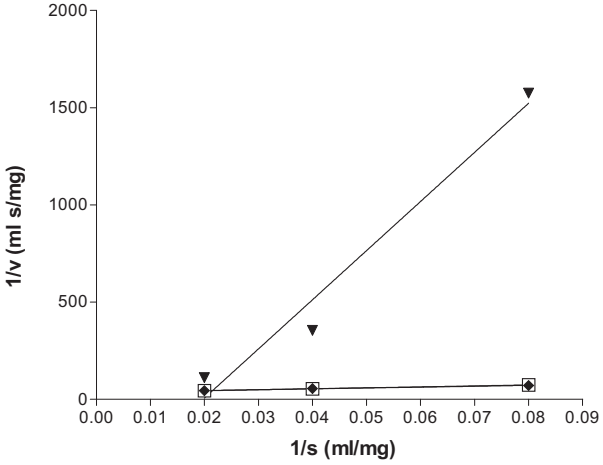

Figure 1 Lineweaver-Burk plots of 1/V *vs.* 1/[S] for amylase hydrolysis of soluble starch and in the presence of lactic acid or tannic acid. -▼- tannic acid ($V_{max} = -2{\cdot}10^{-3}$), -◆- lactic acid ($V_{max} = 0{\cdot}027$), -□- reference ($V_{max} = 0{\cdot}029$).

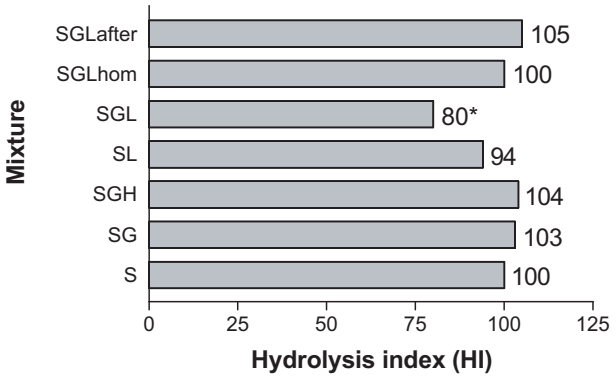

Figure 2 HI for starch/gluten mixtures after different types of processing. S= starch, SG = starch and gluten, SGH = SG and HCl, SL = starch and lactic acid, SGL = SG and lactic acid, SGLhom = SGL homogenised after gelatinisation, SGLafter = SGL lactic acid added after gelatinisation. Values are means ($n = 5$) and values followed by * are significantly different from the others ($P < 0{\cdot}05$).

Bread with lactic acid; impact of homogenisation

The HI of the lactic acid bread was 81, which was significantly lower than the reference bread ($P < 0{\cdot}05$). The HI of the homogenised bread was increased only slightly to 86, which was not significantly different from the HI of the normal lactic acid bread.

Pilot-plant baking of lactic acid bread; influence of protein content of flour and addition of gluten

The HIs of the bread products are shown in Table II. All three products with lactic acid had a significantly lower HI ($P < 0{\cdot}05$) than the reference bread with no acid. Moreover, addition of gluten to a lactic acid bread significantly lowered HI ($P < 0{\cdot}05$).

Meal studies in healthy humans with fermented gruels containing L- or L/D-lactic acid

The postprandial glucose and insulin responses are illustrated in Figures 3 and 4, and GI, II and the corresponding statistics are presented in Table III. The GIs of the L/D-lactic acid gruel and the non-fermented gruel were both significantly higher (162 and 165, respectively) compared with the reference bread meal (GI = 100), whereas the GI of the L-lactic acid gruel (154) did not differ from either the reference bread or the other gruels. The blood glucose response at 30 min was significantly

Table II Hydrolysis index (HI) of bread products baked in pilot-plant equipment

Bread type	HI ± SEM
Wheat bread (flour no. 1)	100[a]
Wheat bread + lac[†] (flour no.1)	80·6 ± 2·5[b]
Wheat bread + lac[†] (flour no. 2)	86·4 ± 2·7[b]
Wheat bread + lac[†] (flour no. 2 + gluten)	72·4 ± 1·8[c]

Values with different superscript letters are significantly different ($P < 0{\cdot}05$), $n = 5$.
[†]Lac; lactic acid.

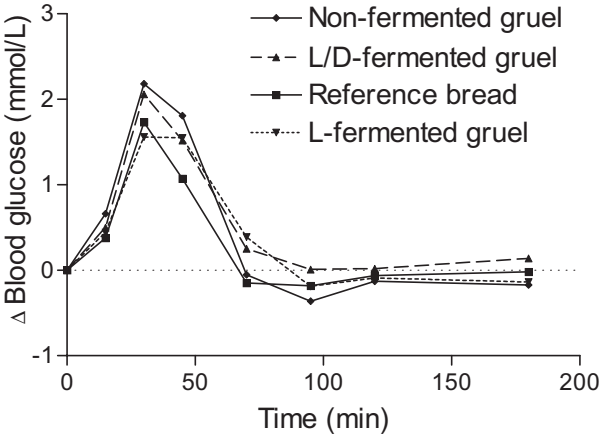

Figure 3 Incremental blood glucose responses in healthy subjects after ingestion of meals containing equal amounts of carbohydrate in a reference bread, a non-fermented barley gruel and fermented barley gruels containing L-lactic acid and L-/D-lactic acid, respectively. Values are means ($n = 10$).

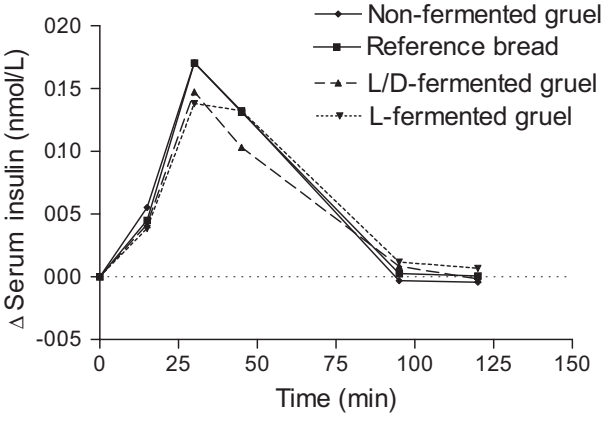

Figure 4 Incremental serum insulin responses in healthy subjects after ingestion of meals containing equal amounts of carbohydrate in a reference bread, a non-fermented barley gruel, and fermented barley gruels containing L-lactic acid and L-/D-lactic acid, respectively. Values are means ($n = 10$).

Table III Postprandial glucose and insulin responses* to a reference bread, a non-fermented gruel, and two lactic acid fermented gruels

Test meal	Glucose response† (Δ mmol/L) 30 min	Glucose response† (Δ mmol/L) 45 min	Glycaemic index 95 min (%)	Insulin response† (Δ nmol/L) 30 min	Insulinaemic index 95 min (%)
Reference bread	$1{\cdot}73 \pm 0{\cdot}2^{a,b}$	$1{\cdot}06 \pm 0{\cdot}2^{a}$	100^{a}	$0{\cdot}17 \pm 0{\cdot}02^{a}$	100^{a}
Non-fermented gruel	$2{\cdot}18 \pm 0{\cdot}2^{a}$	$1{\cdot}81 \pm 0{\cdot}3^{b}$	165 ± 17^{b}	$0{\cdot}17 \pm 0{\cdot}01^{a}$	115 ± 16^{a}
L/D‡-fermented gruel	$1{\cdot}91 \pm 0{\cdot}2^{a,b}$	$151 \pm 0{\cdot}2^{a,b}$	162 ± 21^{b}	$0{\cdot}15 \pm 0{\cdot}02^{a}$	95 ± 13^{a}
L‡-fermented gruel	$1{\cdot}56 \pm 0{\cdot}2^{b}$	$1{\cdot}55 \pm 0{\cdot}3^{a,b}$	$154 \pm 31^{a,b}$	$0{\cdot}14 \pm 0{\cdot}02^{a}$	109 ± 19^{a}

* All values are expressed as mean ± SEM, $n = 10$. Values followed by different letters are significantly different ($P < 0{\cdot}05$).
† Glucose or insulin levels were not statistically different at any other time point than those presented here.
‡ L and D indicate the isomeric form of the lactic acid present in the gruel.

lower ($P < 0{\cdot}05$) after the gruel with L-lactic acid compared with the non-fermented gruel. At 45 min the glucose level after the bread reference meal was significantly lower ($P < 0{\cdot}05$) than the corresponding level for the non-fermented gruel. No differences in insulin levels or IIs were found between the test products (Table III).

DISCUSSION

The results from the meal study with fermented barley gruels suggested that lactic acid, independent of its isomeric form, had no lowering effect on the blood glucose and insulin responses. In contrast, it was possible to achieve a lowered HI with flour/water and starch/gluten mixtures, when lactic acid was added prior to the heat treatment. Consequently, the lack of effect on glycaemia in the case of fermented barley gruels might be due to the 'late' addition of fermentative lactic acid bacteria. This hypothesis was further strengthened by *in vitro* experiments (data not shown), which gave a significant difference ($P = 0{\cdot}03$) in HI between two barley flour/water mixtures, with lactic acid added prior to (HI = 81), respectively after (HI = 89) heat treatment. Possibly, the gastric emptying of gruels is at a higher rate than breads[23], which may counteract a reduced rate of starch digestion. Another probable cause for the lack of lactic acid effect in the gruels might have been that the high amylose barley flour was hydrolysed by the enzyme preparation (*SAN Super*). The protease activity in *SAN Super* might also have eliminated important starch–protein interactions.

The present studies with lactic acid in bread and starch/gluten mixtures suggest that the microstructure, as well as interactions between the lactic acid, starch, and gluten, play important roles for the lowered starch hydrolysis. Gluten alone showed no impact on HI for starch in water. Only when the gluten was combined with lactic acid, an HI-lowering was seen for starch. The lower HI for the starch/gluten mixture with lactic acid added before gelatinisation was not related to a low gelatinisation pH. Thus, no difference was found between the starch/gluten mixture and the reference, when HCl was added to give the same gelatinisation pH as it was with the lactic acid. With soluble starch as substrate, lactic acid did not behave like a classical enzyme inhibitor in the Lineweaver-Burk plot. These findings point to the creation of a barrier to starch digestion caused by heat-treatment in the presence of lactic acid, rather than to an inhibitory effect of lactic acid *per se*.

In the case of lactic acid bread, an even lower HI was attained when extra gluten was added to the dough, compared with a corresponding lactic acid bread with no extra gluten. However, in contrast to the disappearance of the lactic acid effect in the homogenised starch/gluten mixtures, it was not possible to affect the rate of starch hydrolysis in bread by homogenisation. The divergence in results between the bread and the starch/gluten mixture may be due to the components in flour, presence of monoglycerides in the bread, or the difference in water content. In ordinary white wheat bread with added lactic acid, there is a ten-fold higher amount of starch per gram water than in the starch/gluten mixture. Also, the structure of bread is different from the starch/gluten mixture and possibly the interaction between the starch and gluten is too strong to be destroyed by homogenisation. In another study[8], when spaghetti was homogenised, the HI increased compared with intact spaghetti, but did not reach the level seen with bread baked from the identical ingredients.

The impact of gluten on postprandial blood glucose responses has previously been studied in other laboratories. Whereas Jenkins *et al.*[24] found that bread made from gluten-free flour gave a significantly higher blood glucose response in healthy subjects, Packer *et al.* did not find any differences in GI between gluten containing and gluten-free foods[25].

It can be concluded that the inclusion of lactic acid in bread reduces the rate of starch digestion. The current work suggests that the presence of lactic acid during heat treatment, promote interactions between the starch and gluten, hence reducing the starch bioavailability. This is the probable mechanism for the lowered glycaemia seen in bread products with lactic acid. The presence of lactic acid during heat treatment appears to be a prerequisite and no effect was seen when lactic acid was added following simulated baking nor produced post-gelatinisation in a gruel product. In future attempts to create low GI lactic acid bread with good sensory properties, it will be of great interest to evaluate further the optimal proportions between lactic acid and gluten.

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