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SHORT COMMUNICATION

The sourdough fermentation may enhance the recovery from intestinal inflammation of coeliac patients at the early stage of the gluten-free diet

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Abstract
Purpose This study aimed at investigating the effect of corn, rice and amaranth gluten-free (GF) sourdoughs on the release of nitric oxide (NO) and synthesis of pro-inflammatory cytokines by duodenal mucosa biopsies of eight coeliac disease (CD) patients.
Methods Selected lactic acid bacteria were used as starters for the manufacture of corn, rice or amaranth sourdoughs. Chemically acidified doughs, without bacterial starters, and doughs started with baker's yeast alone were also manufactured from the same GF matrices. Pepsin-trypsin (PT) digests were produced from all sourdoughs and doughs, and used to assay the rate of recovery of biopsy specimens from eight CD patients at diagnosis. The release of NO and the synthesis of pro-inflammatory cytokines interferon-γ (IFN-γ) were assayed.
Results During fermentation, lactic acid bacteria acidified and grew well (ca. log 9.0 CFU/g) on all GF matrices, showing intense proteolysis. Duodenal biopsy specimens still released NO and IFN-γ when subjected to treatments with basal medium (control), PT-digest from chemically acidified doughs and PT-digest from doughs fermented with baker's yeast alone. On the contrary, the treatment of all the biopsy specimens with PT-digests from all GF matrices subjected to sourdough fermentation significantly decreased the release of NO and the synthesis of IFN-γ.
Conclusions During manufacture of GF baked goods, the use of sourdough fermentation could be considered as an adjuvant to enhance the recovery from intestinal inflammation of coeliac patients at the early stage of the gluten-free diet.

Keywords Coeliac disease · Sourdough · Nitric oxide · Interferon-γ · Biopsies of duodenal mucosa

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Introduction

Coeliac disease (CD) is an inflammatory disorder of the small intestine that affects genetically predisposed individuals when they ingest gluten from all *Triticum* species and similar proteins of barley, rye and their cross-bred varieties. It is generally accepted that CD is a T-cell-mediated disease, where mainly gliadin-derived peptides, either in native form or in form deamidated by tissue transglutaminase, activate the lamina propria T lymphocytes that release pro-inflammatory cytokines interferon-γ (IFN-γ) [1, 2]. The prevalence of CD is worldwide increasing; it is estimated to be 0.5–2.0% in most of the European countries and the United States [3]. Although alternative therapeutic strategies are currently investigated [4, 5], the dietary therapy is the only accepted treatment for CD. It consists in the life-long strict gluten-free diet (GFD), which is expected to improve the symptoms and nutritional status and to prevent long-term complications [6].

Over the last years, the demand for high-quality and natural-GF baked goods markedly increased [7, 8]. A number of GF flours and nutrient-dense grains, seeds and

legumes offer a considerable potential for the formulation of GF baked goods [6, 9]. Most of these GF baked goods are fermented by baker's yeast alone. Recently, the sourdough, a cocktail of acidifying and proteolytic lactic acid bacteria together with yeasts, was considered as the leavening agent for making GF baked goods [10–12]. The sourdough would be useful for (1) preventing risks of gluten contamination; (2) improving the nutritional properties (e.g. minerals, amino acids); (3) enhancing texture and flavour; and (4) extending the shelf-life by decreasing or eliminating the use of chemical preservatives [10]. High-quality GF baked goods are essential, especially for CD patients who are diagnosed at an advanced age and have difficulties to strictly adhere to GFD. No studies considered the effect of sourdough GF baked goods on the recovery of the intestinal mucosa of CD patients at the early stage of GFD. Although organ culture systems are unlikely to be representative of the events that take place at level of the small intestine of CD patients, duodenal specimens accurately mirror the in vivo conditions. Recent investigations by capsule endoscopy showed that most of the residual changes during GFD remain in the duodenum, thus confirming the potential of studies based on duodenal biopsies [13]. The synthesis of nitric oxide (NO) and pro-inflammatory cytokines is usually found when CD biopsy specimens are in vitro cultured with gluten [14]. The combined release of NO and IFN-γ from CD biopsies is considered a useful tool to investigate the activation and/or deactivation of the local immune response.

This study aimed at investigating the effect of corn, rice and amaranth GF sourdoughs on the release of NO and synthesis of pro-inflammatory cytokines from duodenal mucosa biopsies of eight CD patients.

Methods

Microorganisms

Lactobacillus alimentarius 15M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A and *Lactobacillus hilgardii* 51B were previously selected based on their capacity to hydrolyse gliadins [15]. *L. sanfranciscensis* LS3, LS10, LS19, LS23, LS38 and LS47 were previously selected based on their peptidase activities towards Pro-rich peptides [16]. All lactobacilli were isolated from wheat sourdoughs, which are traditionally used for making typical Italian breads. Strains were propagated for 24 h at 30 °C in MRS broth (Oxoid, Basingstoke, Hampshire, England), with the addition of fresh yeast extract (5%, v/v) and 28 mM maltose at the final pH of 5.6. Cultivation of lactobacilli for making sourdoughs was until the late exponential phase of growth was reached (ca. 12 h).

Sourdough fermentation

The characteristics of corn (*Zea mais* L.), rice (*Oryza sativa*) and amaranth (*Amaranthus hypochondriacus*) flours were as follows: moisture, 11.1, 11.9 and 9.8%; protein (N × 5.70), 6.8, 5.9 and 14.3% of dry matter (d.m.); fat, 2.8, 1.4 and 6.5% of d.m.; ash, 0.6, 0.6 and 3.0% of d.m.; total carbohydrates, 78.7, 80.1 and 66.2% of d.m, respectively. Twenty-four-hour-old cells of lactic acid bacteria were harvested by centrifugation (10,000×*g*, 10 min, 4 °C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0) and resuspended into tap water at the cell density of ca. log 8.0 CFU/ml. To prepare 100 g of sourdoughs (corn sourdough, CS; rice sourdough, RS; and amaranth sourdough, AS), 45.5 g of flours and 54.5 ml of tap water, containing the above cell suspension of each lactic acid bacterium (cell density in the dough of ca. log 8.0 CFU/g), were used. The dough yield, [(dough weight/flour weight) × 100] was 220. Corn, rice or amaranth chemically acidified doughs (C-, R- or A-CAD), without bacterial starters, were acidified to pH 3.3, 2.7 and 3.0, respectively, by a mixture of lactic and acetic acids (molar ratio 1:4). The fermentation was allowed at 37 °C for 24 h, under stirring conditions (ca. 200 rpm). Corn, rice or amaranth doughs were also fermented (1.5 h at 30 °C) by baker's yeast alone (2%, wt/wt) (CBY, RBY and ABY, respectively). All fermentations were carried out in triplicate.

Determination of cell yield, pH, titratable acidity and free amino acids

Cell numbers of lactic acid bacteria were estimated by plating on mMRS agar medium at 30 °C for 48 h. The values of pH were determined online by a pH metre (Model 507, Crison, Milan, Italy) with a food penetration probe. Total titratable acidity (TTA) was determined on 10 g of dough homogenized with 90 ml of distilled water and expressed as the amount (ml) of 0.1 M NaOH to get pH of 8.3. Total and individual free amino acids from the water extracts of doughs were analysed using the Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England), equipped with a Na-cation exchange column (20 by 0.46 cm inner diameter), as described by Rizzello et al. [17].

Protein extraction and pepsin-trypsin (PT) digest

Doughs (100 mg) were suspended on 4 ml of 0.2 N HCl, homogenized with a Sterilmixer Lab (PBI International, Milan, Italy) and added of 2 ml of 0.6 M Tris–HCl, pH 7.4, containing 30% (wt/vol) glycerol, 6% (wt/vol) sodium dodecyl sulphate (SDS) and 6% (vol/vol)

2-mercaptoethanol. The protein concentration of the extracts was determined by the Bradford method, using bovine serum albumin as the standard [17]. Doughs (containing 500 mg of proteins) were subjected to sequential PT hydrolysis to simulate the in vivo digestion [18].

Processing of duodenal biopsies and in vitro organ culture

Duodenal biopsies were obtained from 8 CD patients (5 girls and 3 boys, average age of 5 years) who underwent gastrointestinal endoscopy for diagnostic purposes. Each patient suffered from symptoms suggestive of CD and returned a positive serum transglutaminase antibody. The diagnosis of CD was determined using the Marsh criteria for histopathology (six patients were scored 3, and two patients were scored 2) and the positive serology (endomysial and/or tissue transglutaminase antibodies). No differences were found between girls and boys. The study was approved by the National Institute of Health Committee, Rome, Italy, and written informed consent was obtained from the parents of patients. All patients were on gluten containing diet at the enrolment. Biopsy specimens were from distal duodenum. Histopathological diagnosis of CD was based on typical mucosal lesions with crypt cell hyperplasia and villous atrophy. Biopsy specimens from CD patients were placed in 24-well plates and cultured in the Dulbecco's modified Eagle's medium (DMEM), without phenol red, and supplemented with 10% (wt/vol) foetal bovine serum (FBS), 2 mmol/l glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mg/ml gentamycin (Biowhittaker, Milan, Italy) and 1 mg/ml PT-digest. Incubation was at 37 °C for 48 h, under 95% humidified atmosphere and 5% CO_2. DMEM medium alone was used as the control. After incubation, supernatants were collected and stored at −70 °C until use.

Nitric oxide (NO) and interferon-gamma (IFN-γ) analyses

The level of NO was determined by measuring the stable oxidation products nitrite and nitrate in the organ culture supernatants [19]. The reaction was carried out on 96-well plates. Supernatants were mixed with an equal volume of Griess reagent (Sigma Aldrich, St. Louis MO, USA) (1%, wt/vol, sulphanilic acid in 0.5 M HCl and 0.1%, wt/vol, N^1-1-napthylethylendiamine hydrochloride), and the absorbance at 540 nm was measured after 30 min by a microplate reader (Bio Rad, Hercules, CA). The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

The concentration of IFN-γ of the supernatant of the organ cultures was determined by commercial ELISA kit, according to the manufacturer's instructions (PeproTechEC, NJ, USA).

Statistical analysis

Data were subjected to 2-tailed Wilcoxon signed ranks test. P values lower than 0.05 ($P < 0.05$) were considered statistically significant.

Results

During sourdough fermentation, lactic acid bacteria grew from ca. log 8.1 ± 0.2 CFU/g to log 9.0 ± 0.4, 9.3 ± 0.4 and 9.6 ± 0.5 CFU/g in amaranth (AS), rice (RS) and corn (CS) sourdoughs, respectively. No growth of lactic acid bacteria was found during incubation of corn, rice or amaranth chemically acidified doughs (C-, R- or A-CAD). The cell number was below log 3.0 CFU/g. During fermentation with baker's yeast alone, the cell number of yeasts of corn, rice or amaranth doughs (CBY, RBY and ABY, respectively) did not vary compared with the initial value (ca. log 8.0 CFU/g). Also these doughs did not harbour lactic acid bacteria at cell density higher than log 3.0 CFU/g. The initial value of pH differed between CS, RS and AS. It was 6.2 ± 0.3, 5.83 ± 0.1 and 5.87 ± 0.2, respectively. After sourdough fermentation, the values of pH were 3.3 ± 0.03 (CS), 2.7 ± 0.04 (RS) and 3.0 ± 0.04 (AS). The values of pH of C-CAD, R-CAD and A-CAD were the same (3.3, 2.7 and 3.0) and did not vary during incubation. The values of pH of CBY, RBY and ABY remained almost constant during incubation (ca. 6.0). The value of TTA was the highest for AS (24.4 ± 0.4 ml 0.1 M NaOH 10/g), followed by RS and CS (17.3 ± 0.3 and 11.4 ± 0.2 mL 0.1 M NaOH 10/g, respectively). The concentration of total free amino acids of CS, RS and AS sourdoughs (3,050 ± 25, 856 ± 18 and 7,600 ± 49 mg/kg) was significantly ($P < 0.05$) higher than that found in CBY, RBY and ABY (750 ± 21, 310 ± 20 and 1,710 ± 25 mg/kg). Almost the same values of total free amino acids were found in C-CAD, R-CAD and A-CAD (1,100 ± 26, 430 ± 15 and 2,203 ± 37 mg/kg). Therefore, C-CAD, R-CAD and A-CAD had the same values of pH of CS, RS and AS, but showed a very lower extent of proteolysis.

The release of NO from the biopsy specimens of the eight CD patients was determined after 48 h of incubation with PT-digests from various sourdoughs and doughs (Fig. 1). Since no significant ($P > 0.05$) differences were found between chemically acidified doughs and those started with baker's yeast alone, the results only concern samples chemically acidified. The release of NO from the control DMEM was ca. 4.5 mM per mg of protein. This

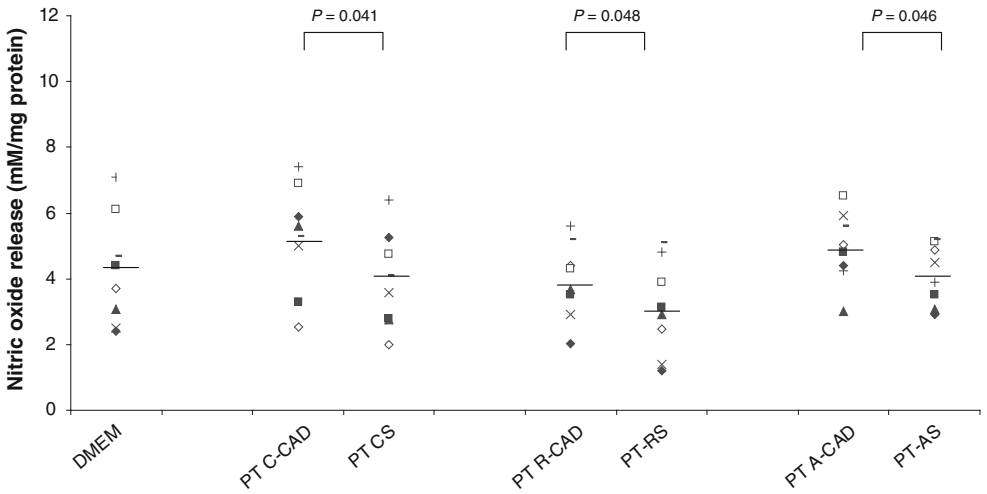

Fig. 1 Nitric oxide release (mM/mg protein) by duodenal biopsies from eight untreated coeliac patients, incubated at 37 °C for 48 h with PT-digests (1 mg/ml) of corn (CS), rice (RS), amaranth (AS) sourdoughs and related chemically acidified doughs (C-, R- or A- CAD). Dulbecco's modified Eagle's medium (DMEM) was used as the control. The median values (*horizontal line*) and *P* values were calculated for each group. *P* values <0.05 were considered statistically significant

showed an obvious inflammatory state of the biopsy specimens at the diagnosis. The comparison between treatments of biopsy specimens with PT-digests from C-, R- and A-CAD or CS, RS and AS showed a significant ($P = 0.068$, 0.111 and 0.056, respectively) decrease in the release of NO. The highest percentages of decrease were found with PT-digests from CS and RS (32.3 and 32.8%, respectively).

IFN-γ is the main cytokine involved in the inflammatory response of CD [20]. To assays the synthesis of IFN-γ, duodenal biopsies were incubated for 48 h with the PT-digests from sourdoughs and doughs (Fig. 2). Also in this case, no significant ($P > 0.05$) differences were found between chemically acidified doughs and doughs started with baker's yeast alone (data not shown). When treated with PT-digests from C-, R- and A-CAD, the synthesis of IFN-γ by biopsy specimens was the same as the negative control DMEM. When duodenal biopsy specimens from coeliac patients were incubated with CS, RS or AS, the synthesis of IFN-γ significantly decreased. The decreases were 68.2 ± 31 (CS) versus 105.2 ± 39 (C-CAD), 67.8 ± 38 (RS) versus 111.0 ± 41 (R-CAD) and 53.0 ± 28 (AS) versus 98.2 ± 40 (A-CAD) pg/ml.

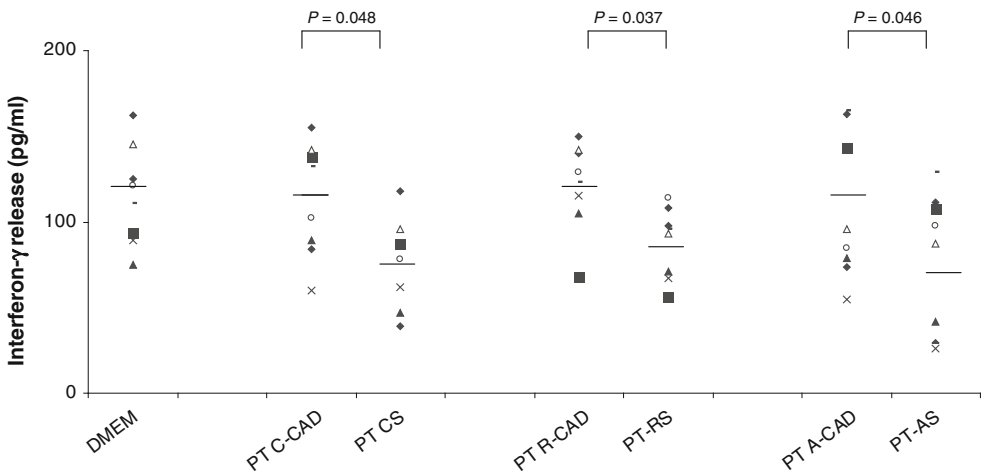

Fig. 2 Interferon-γ release (pg/ml) by duodenal biopsies from eight untreated coeliac patients, incubated at 37 °C for 48 h with PT-digests (1 mg/ml) of corn (CS), rice (RS), amaranth (AS) sourdoughs and related chemically acidified dough (C-, R- or A- CAD). Dulbecco's modified Eagle's medium (DMEM) was used as the control. The median values (*horizontal line*) and *P* values were calculated for each group. *P* values <0.05 were considered statistically significant

Discussion

Nowadays, most of the GF baked goods from the market include corn and rice flours, which are subjected to fermentation by baker's yeast alone. Amaranth flour also deserves an increasing interest because of the high nutritional properties and characteristic flavour [21–24]. Sourdough is an alternative and natural starter for making baked goods, also including GF products [25]. A large number of lactic acid bacteria strains were previously selected aiming at the complete degradation of gluten in wheat flour bread [17, 26]. All these strains show complementary proteinase and, especially, peptidase activities, which completely degraded the epitopes responsible for CD [27]. The selected lactic acid bacteria were used as sourdough starters to ferment corn, rice and amaranth flours. They grew and acidified the GF matrices, showing performance that agreed with those usually found during wheat sourdough processes [28].

Although not specific, the main expression of CD is small intestine lesions that impair nutrient absorption and improve upon withdrawal of the responsible cereals. Because of the high glutamine (>30%) and, especially, proline (>15%) content, wheat (gliadins), rye (secalins) and barley (hordeins) prolamins are toxic, together with some glutenin epitopes, to CD patients. During cereal ingestion, the synthesis of NO at level of the small intestine has a pivotal role on the histological features of CD [14, 29]. As shown for the ulcerative colitis also, the increase in the release of NO is considered to be a marker of the inflammation state [30]. Because of the low proportion of glutamic acid and, especially, proline [31], prolamins of corn and rice are tolerated by CD patients. Nevertheless, the duodenal biopsy specimens of the eight CD patients still released NO and IFN-γ when subjected to treatments with DMEM basal medium (control) and PT-digests from corn, rice and amaranth doughs, which were chemically acidified or fermented with baker's yeast alone. NO synthase (NOS) II, the major inducible isoform of NOS, is mainly expressed by inflammatory cells (e.g. nervous cells, macrophages and enterocytes) during activation by pro-inflammatory cytokines (e.g. IFN-γ) [32, 33]. However, NOS, which is constitutively expressed in human duodenal enterocytes, increased in patients with untreated coeliac disease and partially restored under treatment [33]. The synthesis of IFN-γ is the signature of gluten peptide–specific HLA-DQ2- and HLA-DQ8-restricted T cells that are isolated from the mucosa of the small intestine of CD patients. IFN-γ has a key role in the downstream initiation of mucosal damage [34]. When subjected to sourdough fermentation, the decrease in the release of NO was found for all GF matrices and all eight duodenal biopsy specimens. The biological acidification and, especially, proteolysis by sourdough lactic acid bacteria were responsible for this effect [35]. Chemically acidified doughs having a low level of proteolysis did not act as the sourdough samples. The highest effect was found for corn and rice sourdoughs. PT-digests from corn, rice or amaranth sourdoughs also decreased the synthesis of IFN-γ from duodenal biopsy specimens. In particular, the decrease in IFN-γ was the highest under treatment with PT-digests of amaranth and corn sourdoughs. At least on biopsies of CD mucosa, which were maintained under organ culture, the neutralization of IFN-γ prevented the further gluten-induced mucosal damage [36]. Overall, no relationships were found between the variability of the histopathological score (Marsh grade) of the biopsy specimens and the effect of sourdough fermentation on the release of NO and IFN-γ.

Although preliminary, the results of this study suggested that sourdough fermentation for making GF baked goods may enhance the rate of recovery of the mucosal injury of CD patients at the early stage of GFD.

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