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Biotechnological Treatments of Gluten
and Gluten-Free Flours to Improve Dough Texture Properties
and Aroma Profiles of the Product

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Abbreviations Used

acTG	Activa transglutaminase
AEC	3-amino-9-ethylcarbazole
ANOVA	analysis of variance
AtPng1p	*Arabidopsis thaliana* peptide N-glycanase
BC	biotin-cadaverine
BSA	bovine serum albumin
CD	celiac disease
CFU	colony forming unit
DMC	N', N'-dimethyl casein
DY	dough yield
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immune sorbent assay
FN	fibronectin
GC-MS	gas chromatography-mass spectrometry
GF	gluten-free

HMW	high molecular weight
HRP	horseradish peroxidase
LAB	lactic acid bacteria
MCFA	medium-chain fatty acid
mTG	microbial transglutaminase
NCGS	non-celiac gluten sensitivity
PC	principal component
PCA	principal component analysis
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SPME	solid phase micro-extraction
TG	transglutaminase
TPA	texture profile analysis
VCs	volatile compounds
WM	transglutaminase
YPD	yeast extract peptone dextrose

Introduction

Cereals have a long history of use by humans. They are the most important sources of food, and cereal-based foods are a major source of energy, carbohydrate, protein and fibre, B vitamins and minerals for the world population. For this reason industry of bakery and farinaceous products occupies an important place in the production and market consumption.

Gluten is the major factor involved in the structural properties of bakery products. Thanks to its ability to create aggregates, it gives dough viscosity, elasticity and cohesion, all features responsible for the baking performance (Delcour, Joye, Pareyt, Wilderjans, Brijs, & Lagrain, 2012). Gluten is composed of extensible, viscous gliadins and rigid, elastic glutenins. Moreover, the gliadin protein fraction is the main factor responsible for the development of celiac disease (CD) and other non-celiac gluten sensitivities. The only treatment for these people is lifelong adherence to a strict gluten-free diet (Dowd, Tamminen, Jung, Case, McEwan, & Beauchamp, 2014). In recent years there has been increasing interest on gluten-free breads, mainly involving the approach of incorporation of starches, dairy proteins and hydrocolloids (hydroxypropylmethylcellulose, methylcellulose, carboxymethylcellulose, psyllium gum, locust bean gum, guar gum, and xanthan) into a gluten-free flour base that

could mimic the viscoelastic properties of gluten and result in improved structure, mouthfeel acceptability and shelf-life of these products. There is, therefore, an urgent need to investigate the potential of the bread-making ingredients, additives and technological aids, to develop high-quality gluten-free products.

Nowadays, the development of new technologies in the food industry aims to both improve products already marketed, and to develop new gluten-free products. The replacement of the gluten network in the development of gluten-free bakery products is a challenging task for the researchers of food science. The functionality of proteins from gluten-free flours could be modified in order to improve their baking characteristics by promoting protein networks. Transglutaminase is an enzyme that forms cross-links between protein molecules and it has been successfully used in the food industry to promote protein cross-linking. The application of cross-linking enzyme in gluten-free bakery process modified the viscoelastic properties of the dough, improving the quality of the resulting gluten-free product by promoting protein network. In particular, the effect of transglutaminase treatment improves the structural and rheological properties of the final product.

Moreover, the use of sourdough represents an attractive alternative for increasing the quality of gluten-free breads. Sourdough addition has a strengthened role in improving the quality of gluten-containing bread. Sourdough is a principal means of biological leavening in bread making due to the production of carbon dioxide and other volatile compounds. It consists in a mixture of flour (from cereals) and water, which is fermented by the action of lactic acid bacteria and yeasts. The positive effects associated with the use of sourdough including improvements in bread volume and crumb structure, flavor, nutritional value and mould-free shelf-life. These positive effects are associated with the metabolic activity of the selected pure

cultures used in the composition of the sourdough, e.g. lactic acid fermentation, proteolysis, exopolysaccharide production and synthesis of volatile and antimicrobial compounds (Corsetti, Settanni, Chaves López, Felis, Mastrangelo, & Suzzi, 2007).

Up to date, no systematic studies have been reported on the effect of the combined use of protein cross-linking enzyme and sourdough on the rheological properties of dough and quality of final fresh bread obtained from gluten and gluten-free flours. However, sourdough has been traditionally added in the dough to improve bread quality, and transglutaminase has been studied as a aggregation additives in food system. Thus, we now present the effects of the two biological agents together.

1. General Background

1.1. Baking Technologies

Bread making is one of the oldest technologies known to mankind. Baked product, in form of bread is consumed in large quantity in the world in different types and form depending on cultural habits and geographical area. Its production techniques and the products differ widely around the world. Essentially, the production of bread requires three important ingredients such as flour, yeast and water. The main objective of bread making is to convert cereal flours into attractive, palatable, and digestible food.

Wheat flour is the most common used in bread making for its unique technological characteristics. The principal features of wheat bread quality are high volume, soft and elastic crumb structure, good shelf-life and microbial safety of the product (R. S. Chavan & Jana, 2008). Wheat-bread formulation contains the essential structure-building proteins, that contributing to the appearance, crumb structure, and consumer acceptability of baked products. Even so, bread made from unconventional flours are also available.

1.1.1. Gluten and Viscoelastic Properties of Dough

Flour is the most important ingredient in bread making because it modulates the specific characteristics of bakery products. It consists of protein, starch and other carbohydrates, ash, fibers, lipids, water, and small amounts of vitamins, minerals, and enzymes.

The most important proteins in baking technology, that wheat flour contains are gliadin and glutenin, classified according to their solubility in alcohol/water solution. Gliadin fraction is composed of monomeric proteins with a molecular weight of 30–80 kDa and can be further classified into four groups (α, β, γ and ω) (Song & Zheng, 2007; van den Broeck, America, Smulders, Bosch, Hamer, Gilissen, et al., 2009). The glutenin fraction comprises aggregated proteins linked by inter chain disulphide bonds. Based on primary structure, glutenin subunits have been divided into the high-molecular weight glutenin subunits and low-molecular weight glutenin subunits of 12–60 and 60–120 kD respectively (Song & Zheng, 2007; van den Broeck, et al., 2009). The molecular weight distribution is determined by the ratios of monomeric/polymeric proteins and glutenin/gliadin (Southan & MacRitchie, 1999).

When, during the dough preparation their mixture, known as gluten, is wetted they form a cohesive and elastic network, which gives to wheat dough its unique functional properties (R. S. Chavan & Jana, 2008).

Non-covalent bonds, such as hydrogen bonds, ionic bounds, and hydrophobic bounds, are important for the aggregation of gliadins and glutenins and implicate structure and physical properties of dough. Gliadins are responsible for dough's cohesiveness, while the glutenins are apparently responsible for the dough's resistance to extension (Hoseney, 1994).

Indeed, gluten is the main structure-forming protein present in wheat flour, and plays a major role in bread-making functionality by providing viscoelasticity to the dough, good gas-holding properties, and contributes to the appearance and crumb structure of many baked products (Moore, Schober, Dockery, & Arendt, 2004).

Gluten removal results in major problems for bakers, and currently, many gluten-free products available on the market are of low quality, exhibiting poor mouth-feel and flavour.

The quantity and quality of gluten proteins largely determine dough mixture requirements and the rheological properties of the dough. Furthermore, they contribute to the gas retention properties of the fermenting dough, which determine loaf volume and crumb structure of the resulting bread.

1.1.2. Gluten Sensitive People and Gluten-Free Baked Products

Growing number of people are being diagnosed with coeliac disease, due to improved analytical methods. Epidemiological studies have shown that the prevalence of this lifelong disorder lie between 0.40 and 0.75% in Europe, with a tendency towards higher values (Catassi & Fasano, 2008).

Celiac disease is a chronic inflammatory disorder characterized by damage of the small intestinal mucosa, which destroys mature absorptive epithelial cells on the surface of the small intestine. Gliadin fraction of wheat gluten, and similar alcohol-soluble proteins (prolamine) of barley and rye, are the main responsible of the disease in genetically susceptible individuals (Fasano & Catassi, 2001; Mäki & Collin, 1997). The symptoms can vary greatly and are different in children and adults. But the

clinical presentations ranging from digestive system disorder, such as diarrhea, nutrient malabsorption and weight loss, and also not digestive related signs.

Celiac patients usually present high levels of circulating immunoglobulin A (IgA) antibodies directed to different antigens, in particular tissue transglutaminase, gliadin, and endomysium, whose presence is correlated with gluten dietary intake.

Several studies reported that gluten intolerance can also affect people who do not suffer from celiac disease. Non-celiac gluten sensitivity (NCGS) is a new syndrome of gluten intolerance. The first reports about this gluten-related disorder demonstrate that there is a group of patients whose symptoms have disappeared with gluten withdrawal from diet (Cooper, Holmes, Ferguson, Thompson, Allan, & Cooke, 1981). NCGS can be diagnosed in those patients with gluten intolerance who do not develop antibodies that are typical of celiac disease.

These gluten-related diseases, increasingly diagnostized throughout the world, can only be controlled by maintaining a strictly gluten-free diet. To date, these disorders are belonging to the most common food intolerances.

Since wheat is one of the main components of a daily diet worldwide, as well as the basic ingredient of most baked goods, a gluten-free diet is complex and can easily overwhelm patients (Kupper, 2005).

In recent years there has been increasing interest on gluten-free breads; rice, maize, sorghum, millet, teff buckwheat, amaranth, quinoa and also legume flours are suitable for gluten sensitive patients.

Due to the lack of the gluten network, bread formulation obtained with gluten-free flours often results in a liquid batter, rather than a dough system during the pre-baking phase, and can result in baked bread characterized by crumbling texture, poor color and other quality flaws (Gallagher, Gormley, & Arendt, 2003).

The bakery products available in today's gluten-free market are characterised by lower palatability than their conventional counterparts and may lead to nutritional deficiencies of vitamins, minerals and fibre. Thus, the production of high-quality gluten-free products has become a very important socio-economical issue.

In baking applications the absence of wheat gluten poses a challenge to maintain good sensory quality, especially bread structure and/or retention of softness during storage (Axel, Röcker, Brosnan, Zannini, Furey, Coffey, et al., 2015; Wolter, Hager, Zannini, Czerny, & Arendt, 2014).

Complex recipe formulations, using different chemical agents (i.e. polymeric substances such as xanthan gum and hydroxypropyl-methyl cellulose) (Moore, Schober, Dockery, & Arendt, 2004) are necessary to mimic the structure-building, viscoelastic and water binding properties of gluten, allowing improvement of structure, mouth-feel, acceptability and shel-life of these products.

Furthermore, the functionality of proteins from gluten-free flours could be modified by enzyme action, in order to promote protein networks and improve their baking characteristics. Although proceedings in gluten-free bread formulations have been achieved due to new ingredients and additives.

However, the use of bio-chemical agents presents several disadvantages such as excessive prices (Moroni, Dal Bello, & Arendt, 2009), other allergic reactions (Ortolani & Pastorello, 2006) and predominantly, the use of additives does not match the actual consumers' requirements for natural products (Zannini, Pontonio, Waters, & Arendt, 2012).

1.1.3. Gluten-Free Flours from Cereal and Alternative Source

Interest in the utilization of alternative gluten-free grains for the production of cereal-based foods is growing constantly, mainly due to the rising incidence of gluten sensitive people. As a result, the trend for ethnic and ancient grains has increasingly attracted bakery industries as well as consumers worldwide. The increasing demand of gluten-free food products from consumers has triggered food technologists to investigate a wide range of gluten-free ingredients from different sources to reproduce the unique network structure developed by gluten in a wheat dough system. There are a number of cereals available, which do not contain gluten and are therefore safe to use even by sensitive patients.

Thanks to their abundance in regard to bio- and techno- functional substances and their lack of allergenic proteins, the attention has been focused on novel application of alternative cereal grains, pseudo-cereals, and legumes flours. Their nutritional profile may also counteract the lack of nutrients commonly highlighted in commercial gluten-free bakery and pasta products, providing valuable sources of protein, dietary fiber, vitamins, minerals, and complex carbohydrates, which in turn have a positive impact on human health.

During the last years, novel products appeared on the market labelled with proposed healthier and more natural features compared to their classic wheat counterparts. However, gluten-free bread production results in major challenges for bakers and cereal technologist.

1.2. Transglutaminase

Transglutaminase (TG) is protein-glutamine γ-glutamyl-transferase (EC 2.3.2.13), belongs to the class of transferases (Marx, Hertel, Pietzsch, & 2008, 2008;

Trespalacios & Pla, 2007). It catalyses the formation of an isopeptide bond between the group of γ-carboxamides of peptide-bound glutamine residues (donor) and a variety of primary amines groups of proteins (acceptors) by an acyl-transfer reaction (Motoki & Seguro, 1998). If lysine is the acceptor of acyl, the two peptide chains are covalently linked through an e-(γ-glutamyl)lysine bond that induce the process of cross-linking, i.e. the formation of inter- or intra-molecular cross-links ε-(γ-Glu)Lys (Folk & Finlayson, 1977; Kashiwagi, Yokoyama, Ishikawa, Ono, Ejima, Matsui, et al., 2002). Thus, the enzyme is capable of introducing covalent cross-links between proteins (Nonaka, Tanaka, Okiyama, Motoki, Ando, Umeda, et al., 1989). In the absence of primary amine groups in the reaction system, water becomes the acyl-acceptor and the γ-carboxy-amide groups of glutamine residues are deamidated, becoming glutamic acid residues (Ando, Imamura, Owada, Kakunaga, & Kannagi, 1989), Figure 1.1.
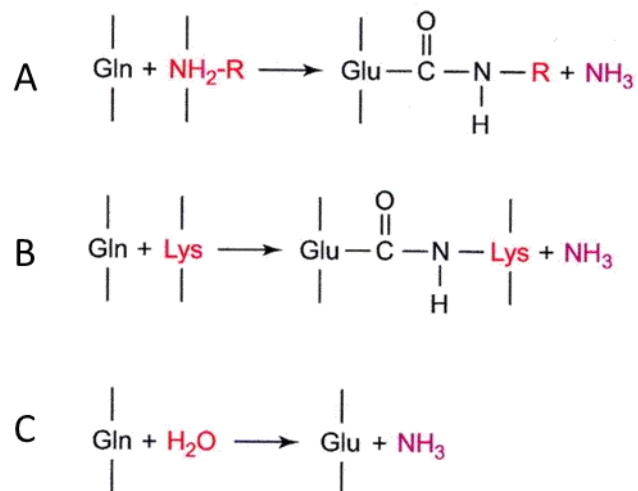
Figure 1.1 The reactions catalysed by transglutaminase included, A: transamidation; B: cross-linking reaction between Gln and Lys residues of proteins or peptides, and C: deamidation.

Transglutaminases are widespread in nature (Kashiwagi, et al., 2002). Multiple molecular forms of transglutaminase are known to play different biological roles inside and outside the cells (Lorand & Graham, 2003), representing a large family of

enzymes occurring not only in many tissues and body fluids of mammals but also in invertebrates, plants and microbial cells (Griffin, Casadio, & Bergamini, 2002). It has been demonstrated to be involved in many physiological processes: in coagulation, in antibacterial immune reactions and in photosynthesis (Kashiwagi, et al., 2002).

In higher organisms, transglutaminases play important roles in various biological functions by selectively cross-linking proteins. Eight distinct transglutaminase isoenzymes have been identified in different animal tissues, and some of these have been purified and characterized at molecular level (Aeschlimann, Koeller, Allen-Hoffmann, & Mosher, 1998; Griffin, Casadio, & Bergamini, 2002; Lorand & Graham, 2003). Among the members are factor XIIIa, which stabilizes fibrin clots; keratinocyte transglutaminase and epidermal transglutaminase, which cross-link proteins on the outer surface of the squamous epithelium (Greenberg, Birckbichler, Rice, & J., 1991); and transglutaminase 2 (TG2), which shapes the extracellular matrix, promotes cell adhesion and motility, and is involved in pathogenesis of celiac disease (Akimov, Krylov, Fleischman, & Belkin, 2000; Klock, Diraimondo, & Khosla, 2012).

The biological functions of bacterial transglutaminases remain largely unknown. It has been postulated that *Streptomyces mobaraensis* transglutaminase cross-links inhibitory proteins during the development of aerial hyphae and spores.

Microbial transglutaminase catalyses the same reaction as mammalian; however, it is not homologous to eukaryotic transglutaminase, including TG2, neither in its primary, secondary, nor tertiary structure. Furthermore it is not regulated by calcium (Yokohama et al. 2004) or guanosine-5'-triphosphate (GTP) and has a broader substrate specificity and lower deamidation activity (Kashiwagi, et al., 2002; Ohtsuka, Umezawa, Nio, & Kubota, 2001).

In plant, several transglutaminase activities have been detected both in higher and lower plants, supporting the presence of this type of enzyme. Indeed, this type of activity has been found in various cell compartments, such as chloroplasts, mitochondria, cytoplasm, and cell walls. And the enzyme roles are supposed to contribute to structural or conformational modification processes. Plant transglutaminase' substrates are found in chloroplasts in which light-dependent plastid suggest the involvement of the enzyme in the photosynthesis or photo-protection reactions (Serafini-Fracassini, Del Duca, Monti, Poli, Sacchetti, Bregoli, et al., 2002).

1.2.1. Microbial Transglutaminase

The extremely high costs of manufacturing transglutaminase from animal origin have prompted scientists to search for new sources of this enzyme.

In 1989, the microbial transglutaminase (mTG) isolated from the *Streptoverticillium sp.* strain was first described. Microbial transglutaminase catalyse cross-linking of protein bound glutamine and protein bound lysine, i.e., the typical transglutaminase reaction. Transglutaminase, isolated from *Streptoverticillium mobaraens* is constituted by a single polypeptide chain of 331 amino acids, MW of 37.9 kDa, with an isoelectric point at pH 8.9 (Abd-Rabo, El-Dieb, Abd-El-Fattah, & Sakr, 2010). Its overall structure consists of a compact domain and Cys-64, the residue essential for the catalytic activity, located at the bottom of a deep cleft (Kashiwagi, et al., 2002). A temperature of 40 °C, pH 5.5 is the optimum for the catalytic activity of enzyme. Transglutaminase from *Streptomyces mobaraensis* is probably the most well characterized transglutaminase; however, other microbial transglutaminases have been identified and their production and manufacturing reviewed elsewhere. Indeed,

microbial transglutaminases were discovered in an attempt to find transglutaminases that would be cost-effective for food industry applications.

1.2.2. Transglutaminase and its Application in Food Industry

Various strategies have been explored in the last 20 years to modify the functional properties of proteins and, among these the cross-linking enzyme transglutaminase is attracting an increasing attention as a simple and safe means for protein processing. Transglutaminase, through its ability to produce either isopeptide bonds between endo-protein Gln and Lys residues or γ-glutamyl derivatives with compounds containing primary amino groups has been shown to modify functional properties of both peptides (Esposito, Mancuso, Calignano, Di Pierro, Pucci, & R., 1995) and proteins from different origin (Di Pierro, Rossi Marquez, Mariniello, Sorrentino, Villalonga, & Porta, 2013; Færgemand, Otte, & Bruun Qvist, 1998; Giosafatto, Rigby, Wellner, Ridout, Husband, & Mackie, 2012; Mariniello, Porta, Sorrentino, Giosafatto, Rossi Marquez, Esposito, et al., 2014; Sorrentino, Giosafatto, Sirangelo, De Simone, Di Pierro, Porta, et al., 2012). With respect to applications in food industry the independence of Ca^{2+} is the major advantages of microbial transglutaminase compared to eukaryotic transglutaminase, which need Ca^{2+} for their catalytic activity.

Proteins processed are subjected to significant chemical modification such as change in protein conformation and functional characteristics, including thermo stability, solubility, elasticity and resilience, hydration degree and gelation properties. Transglutaminase has considerable potential to improve the firmness, viscosity, elasticity and water-binding capacity of food products. The results of these modification might exert a considerable impact on the development of new types of

foods with better structural characteristics (Giosafatto, Rigby, Wellner, Ridout, Husband, & Mackie, 2012).

Microbial transglutaminase' features, such as the sequence difference with mammalian enzyme and the independence of Ca^{2+}, together with that of a higher reaction rate, are extremely favourable for the exploitations of such molecular form of transglutaminase as a versatile reagent for protein modification in vitro and, consequently, as a biotechnological tool, especially for industrial applications. Furthermore, microbial transglutaminase does not require activated substituents or additional reagents and, therefore, it provides a simple and safe method for coupling small or high molecular weight molecules to protein substrates. In fact, cross-linking reaction catalysed by microbial transglutaminase takes place at the enzyme active site offering greater selectivity compared with the one involving tyrosinase, which generates a reactive intermediate that undergoes a non-enzymatic coupling reaction.

Therefore, even though microbial transglutaminase has been extensively used so far mostly to improve the physical and textural properties of several protein-rich foods (Zhu, Rinzema, Tramper, & Bol, 1995), this enzyme is now receiving increasingly considerable attention as a tool to couple macromolecules and generating cross-linked networks.

At last, transglutaminase, because of its unique properties, is an enzyme that is widely used in many branches of the food industry. Further research and development towards cost-efficient production of transglutaminase by microorganisms may result in the development of more accessible products with a wider scope of use. Today, transglutaminase is mainly used in meat, fish, dairy, and baking industries.

1.2.3. Effect on the Quality of Bakery Products

In the baking industry, transglutaminase is used to improve the quality of flour, the texture and volume of bread (Moore, Heinbockel, Dockery, Ulmer, & Arendt, 2006) and the texture of pasta after cooking (Kuraishi, Sakamoto, Yamazaki, Susa, Kuhara, & Soeda, 1997). From a nutritional point of view, rice flour contains many valuable nutrients, for instance protein, fibre and vitamins E and B; however, its use is limited to non-fermented bakery products. Research conducted by Gujral and Rosell (2004) has demonstrated that the addition of transglutaminase to rice flour improved the rheological properties of dough by increasing the triglyceride content (Gujrala & Rosell, 2004).

Transglutaminase are currently being used in baking technology to form links between polypeptide prolamin chains. The first data on the baking of pastry with the addition of transglutaminase were provided by Gottmann and Sprossler (1992). Furthermore, transglutaminase was found to have a positive impact on the stability and volume of dough as well as on the improvement of the baking quality of poor flour and, consequently, the texture of the bread (Marco & Rosselli, 2008). Losche (1995)[1] reported that transglutaminase improved the rheological properties of dough and ensured proper pore size and bread elasticity after baking. In addition, transglutaminases were shown to improve water adsorption by dough (Kuraishi, Yamazaki, & Susa, 2001). Modification of wheat flour proteins with transglutaminase increases the elasticity and resilience of dough as well as the volume of bread by 14% in comparison with pastry made from traditionally prepared dough (Gerrard, Fayle, Brown, Sutton, Simmons, & Rasiah, 2001). Recently, the application of

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[1] Losche IK (1995) Enzymes in baking. The World of Ingredients May–June 22–25

transglutaminase in gluten-free systems modified the viscoelastic properties of the batters, improving the quality of the resulting gluten-free breads by promoting a protein network (Gujrala & Rosell, 2004; Moore, Heinbockel, Dockery, Ulmer, & Arendt, 2006). Nevertheless, in these studies xanthan gum and hydroxypropyl-methyl cellulose were added in order to compensate for the absence of the gluten network. However, no investigations were conducted on the impact of the enzyme on the microstructure of the doughs and breads.

Concerns were raised about the usage of microbial transglutaminase for flour modulation due to the role of transglutaminase in the deamidation reaction of gluten peptides in the etiology of celiac disease. Deamidated gluten peptides are known to increase immune-reactivity to gluten peptides in celiac patients (Sollid, 2000). Interestingly, recent data suggest that the cross-linked gluten flour has lower immune-reactivity in a rabbit model system, suggesting that the lower deamidation rate of microbial transglutaminase relative to mammalian transglutaminase, together with the cross-linking of gluten peptides, might potentially reduce this risk (Gianfrani, Siciliano, Facchiano, Camarca, Mazzeo, Costantini, et al., 2007; Zhu & Tramper, 2008).

This background attest that transglutaminase is versatile tool in modern research and biotechnology.

1.3. Sourdough

Sourdough is a mixture of flour and water that is fermented by the action of metabolically active microorganisms. The use of the sourdough as the natural starter for leavening is one of the oldest biotechnological processes in food production (Röcken & Voysey, 1995). It is an intermediate product for dough and bread

preparation and contains lactic acid bacteria and yeast. The lactic acid bacteria that develop in the dough may originate from selected natural contaminants in the flour or from a starter culture containing one or more known species of lactic bacteria. Although these microorganisms originate mainly from flours and process equipment, the resulting composition of the sourdough microbiota is determined by endogenous (e.g. chemical and enzyme composition of the flour) and exogenous (e.g. temperature, redox potential, dough yield and time of the fermentation process) factors.

Microbiological studies have revealed that more than 50 species of lactic acid bacteria, mostly species of the genus *Lactobacillus*, and more than 20 species of yeasts, especially belonging to the genera *Saccharomyces* and *Candida*, occur in mature sourdough. The sourdough microflora is composed of stable associations of lactobacilli and yeasts, in particular due to metabolic interactions.

The sourdough fermentation can be performed as firm dough or as a liquid suspension of flour in water. This proportion between flour and water is called the Dough Yield and is defined as: (amount of flour + amount of water) × 100/amount of flour.

Based on the technology used in artisanal and industrial processes, Böcker et al. (1995) have classified the sourdough in three type (I, II, and III)(Böcker, Stolz, & Hammes, 1995). Type I sourdough is manufactured with a traditional technique and is characterized by continuous (daily) refreshments to maintain the microorganism in an active state, as indicated by their high metabolic activity. In type II sourdough the microorganisms are in the late stationary phase of growth and exhibit restricted metabolic activity. Generally this kind of sourdough is used as acidifier. Finally, type

III is a dried sourdough in powder form, that is used as acidifier supplement and aroma carrier during bread making.

Beyond this classification, the use of sourdough in bread making provide a wide regional variety of bakery products. In fact, many wheat breads are original to the Mediterranean countries, the San Francisco bay, and Southern America, whereas numerous bakery preparations made with mixed flours are typical for Germany, Central and Eastern Europe (De Vuyst & Neysen, 2005). In Italy, sourdough is used for made traditional sweet leavened baked products (e.g. Panettone, Pandoro, and Colomba) and typical Italian bakery products such as flat leavened breads, and snacks for breakfast. Due to the superior sensory quality and the prolonged shelf-life of the resulting baked goods, sourdough processes have retained their importance in modern baking technology.

1.3.1. Properties and Functions of Sourdough

Sourdough plays an important role in the preparation of bread dough to favour technological properties. Compared with other leavening agents (e.g. conventional baker's yeast, *Saccharomyces cerevisiae*), it improves dough characteristic, such as the texture and flavour, besides nutritional value and extended shelf-life of baked products. Due to their ability to produce carbon dioxide and other volatile compounds, lactic acid bacteria and yeast in the sourdough are responsible also for the leavening capacity of bread dough. In particular, depending on the level of lactic acidification, the use of sourdough leads to an increase in bread volume (Clarke, Schober, & Arendt, 2002; Corsetti, Gobetti, De Marco, Balestrieri, Paoletti, Russi, et al., 2000; Crowley, Schober, Clarke, & Arendt, 2002). Texture analyses, reported by Di Cagno et al. (2002) showed a decrease in dough resistance to extension and an

increase in both extensibility and degree of softening before baking (Di Cagno, De Angelis, Lavermicocca, De Vincenzi, Giovannini, Faccia, et al., 2002).

The generation of aroma compounds in sourdough is influenced by the metabolic activity of the microorganisms. Factors influencing their activity, such as temperature and water content, influence the amount of the metabolites formed. In particular, Spicher and Nierle (1984) have found that an increase fermentation temperature increase the total amount of amino acids in a rye sourdough, while the dough yield seems to have a lesser effect (Spicher & Nierle, 1984).

The generation of sufficient amounts of aroma compounds, during fermentation needs a multiple step process of about 12-24 h (Hansen & Schieberle, 2005). The fermentation of soluble carbohydrates (e.g. maltose, glucose, and fructose), metabolism of nitrogenous compounds and generation of volatile compounds by sourdough lactic acid bacteria influence the flavour of baked goods. And results in an increase of free amino acids (Gobbetti, 1998), which contribute directly to flavour or are further subjected to chemical conversion during baking or enzymatic catabolism thus leading to the synthesis of flavour volatile compounds. Alcohols, aldehydes, ketones, esters, ether derivates, furan derivates, hydrocarbons, lactones, pyrazines, pyrrol derivates and sulphur compounds are the flavor stimuli in baked goods (Schieberle, 1996).

During the microbial fermentation, lactic acidification stabilize or increase levels of bioactive compounds. Several studies are reported the potential mechanism by which sourdough fermentation may influence the nutritional quality of the bread. In particular, it can improve the nutritional quality and beneficial effect of food by decreasing or increasing levels of compounds, and enhancing or retarding the bioavailability of nutrients (Poutanen, Flander, & Katina, 2009). Lactic acidification

increases mineral bioavailability as the result of phytate degradation during the sourdough process (De Angelis, Gallo, Corbo, McSweeney, Faccia, Giovine, et al., 2003; Lopez, Krespine, Guy, Messager, Demigne, & Remesy, 2001).

Acidification has been reported to contribute to extended shelf-life through an inhibitory effects on endospore germination and growth of *Bacillus spp*. Besides various compounds (e.g. organic acids, hydrogen peroxide, diacetyl), sourdough may inhibit the growth of other related microorganism. Moreover, antifungal metabolites (e.g. cyclic dipeptides, phenyllacetic acid, 3-hydroxylated fatty acids) are synthesized during fermentation (Schnürer & Magnusson, 2005).

In conclusion, sourdough fermentation has a number of beneficial effects that include prolonged shelf-life, accelerated volume gain, delayed staling, improved bread flavor, and good nutritional value. Sourdough also improves sensory characteristics such as loaf volume, evenness of baking, color, aroma, taste, and texture of bread (R. S.; Chavan & Chavan, 2011).

1.3.2. Starter Cultures and Fermented Products

The sourdoughs started with lactic acid bacteria or with a complex biological association of lactic acid bacteria and yeasts. As a general rule, specific adapted lactic acid bacteria are the predominant microorganisms; occurring cell densities exceeding 10^8 colony forming units (CFU)/g of dough. The principal species isolated from sourdough or used as sourdough starter belonging to 4 genera of lactic acid bacteria: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella*. The highest number of different species is found in the genus *Lactobacillus*.

Lactobacillus are classified on the basis of fermentative types in: obligate homo-fermentative, facultative hetero-fermentative, and obligate hetero-fermentative strains (R. S.; Chavan & Chavan, 2011).

Yeast are associated with lactic acid bacteria in sourdough process. They belong to more than 20 species and the majority appertain to the species *Candida milleri*, *C. holmii*, *S. exiguous* and *S. cerevisiae* (R. S.; Chavan & Chavan, 2011). The yeast/LAB ratio is generally around 1:100 (Gobbetti, Corsetti, & Rossi, 1994). The variability in the number and type of microorganisms species in sourdough affecting, by many factors the formation of fermented metabolites. In addition, the microbial composition of complex biological ecosystem is affected by environmental factors, such as dough hydratation, the type of cereal-flour used, the leavening temperature, and the sourdough maintenance temperature (Gobbetti, Corsetti, & Rossi, 1994).

Sourdough and bread quality are influenced on the basis of the metabolic pathway of the lactic acid bacteria. Homo- and hetero-fermentative metabolism differ fundamentally with respect to the carbohydrate metabolism. In wheat flour, the main available carbohydrate are maltose followed by sucrose, glucose, and fructose. Starting from glucose, homo-fermentative lactic acid bacteria mainly produce lactic acid through glycolysis (homo lactic fermentation), while hetero-fermentative lactic acid bacteria (e. g. *L. sanfranciscensis*) also produce, besides lactic acid, CO_2, acetic acid, and/or ethanol through the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (hetero lactic fermentation). Hexoses other than glucose enter these major pathways at the level of glucose-6-phosphateor fructose-6-phosphate after isomerization and/or phosphorylation. The yeasts present in sourdoughs are not able to ferment maltose.

However, yeasts cell can nevertheless develop because of glucose released into the dough by some lactic acid bacteria species (R. S.; Chavan & Chavan, 2011).

The degradation of proteins is of crucial importance for bread flavor, volume, and texture. The hydrolysis of peptides by sourdough lactobacilli accumulates amino acids in dough in a strain dependent manner, whereas yeasts decrease amino acids levels in dough. Microbial acidification and the reduction of disulphide bonds in dough proteins by hetero-fermentative lactobacilli increase the solubility of proteins and make them more susceptible for proteolytic degradation. Free-amino acids constitute substrates for microbial conversions or are converted to flavor compounds during baking.

The metabolism of volatile compounds during sourdough fermentation is influenced by the interaction between lactic acid bacteria and yeast and the type of fermentative mechanism involved; homo-fermentative lactic acid bacteria mainly synthesized diacetyl, acetaldehyde, and hexanal, while hetero-fermentative strains are characterized by the production of ethyl-acetate, alcohols, and aldehydes. Iso-alcohols (2-methyl-1-propanol, 2,3-methyl-1-butanol), with their respective aldehydes and ethyl-acetate, are characteristics volatile compounds of yeast fermentation (Damiani, Gobbetti, Cossignani, Corsetti, Simonetti, & Rossi, 1996).

1.3.3. Technological Functionality of Sourdough Application

The first reasons for use of fermentation in baking were leavening, flavour formation and improved stability. Sourdough is established technology in improving and diversifying the sensory and nutritional quality of bread (Poutanen, Flander, & Katina, 2009). Now a day, the use of sourdough is extended in baking of gluten-free bread has been efficient in improving product texture and to delay staling (Moore,

Juga, Schober, & Arendt, 2007). Furthermore, the improvement in flavor due to sourdough addition is of particular interest for unconventional-flour bread production. The flavor of the bread can be influenced by the type of starter cultures used, and characteristic flavors are obtained from organic acids and amino acids released during fermentation.

Sourdough has also been studied for gluten degradation to render it suitable for celiac persons. The degradation of the cereal proteins in wheat and rye sourdough fermentation is an acidity related phenomenon, which strongly affects the flavour and texture of bread. Acidification and the reduction of disulphide bonds of gluten by hetero-fermentative lactobacilli increase the activity of cereal proteases and substrate accessibility; amino acids are accumulated by action of strain-specific intracellular peptidases of lactobacilli (Gänzle, Loponena, & Gobbetti, 2008). Thus, proteolysis by lactic acid bacteria has been suggested as a tool for removing gluten and enhancing the nutritional properties of gluten-free bread (De Angelis, Coda, Silano, Minervini, Rizzello, Di Cagno, et al., 2006; Di Cagno, Rizzello, De Angelis, Cassone, Giuliani, Benedusi, et al., 2008; Gänzle, Loponena, & Gobbetti, 2008).

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2. The project

The doctorate program was focused on the study of a new biotechnological process based on the combined use of a protein cross-linking enzyme and a selected microbial consortium as a new tool for cereal flours product.

The project starts from the collaboration of two departments of the Bologna University; the Department of Biological, Geological and Environmental Science (BiGeA), and the Department of Agricultural and Food Science (DISTAL).

The task of the research project was inserted in a bigger contest whose name is "New technology for the made in Italy", and part of the work was supported by funds from the project *New technological approaches for the increase of shelf-life and of the service content in the products related to Mediterranean diet* (ATENA).

During the PhD program new partnership and collaborations have been developed both in national and international contests. In particular, the Department of Medical and Surgical Sciences of the Bologna University, and the Department of Molecular Biology of the Salzburg University, were get involved.

The experimental activities were conducted primarily at the Molecular Biology Lab. of prof. Stefano Del Duca (in Bologna). Specific activities were conducted at the Food Microbiology Lab of prof. Rosalba Lanciotti (in Cesena), and at the Immunology Lab of Dr. Gabriele Gadermaier (in Salzburg).

2.1. Aim of the Research

This project is focused on the improvement of gluten and gluten-free bakery products using a new biotechnological process based on the combined use of a protein cross-linking enzyme (microbial transglutaminase from *Streptoverticillum mobaraense*) and a selected microbial consortium of lactic acid bacteria and yeasts (i. e. selected strains of *Lactobacillus sanfranciciensis* and *Candida milleri*).

The transglutaminase enzyme is studied for its ability to organize and create protein networks allowing an improvement of the structure and texture of the dough made from different flours. The microbial consortium is selected for its capacity to produce interesting metabolites which leads effect on the sensory, rheological and shelf-life features of the final product.

The aim of this study is to evaluate the ability of cross-linking enzyme and sourdough in improving the biotechnological process in order to enhance the protein aggregation and structural stability in gluten and gluten-free flours as well as in baked products.

The novelty behind the rational plan is the evaluation of the consequence of the two biological agents in combination, exploiting the beneficial and technological effects of both. Even the use of different plant sources as a substrates, belong to cereals, pseudo-cereals and legumes allowing us to well understand significant effects in the process.

At molecular level, the proteins rearrangement after enzymatic treatment, was investigated by sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) and biochemical colorimetric tests. These analyses were performed both on the total protein components and on three selected fractions corresponding to albumins/globulins, prolamins and glutelins.

The microbiological analysis was conducted in order to study the adaptability of the microbial consortium to sourdough prepared from unconventional flours. Bacteriological cultures and microbiological methods were used. In order to evaluate the sourdough metabolic activity and the fermentation process, volatile compounds were detected and analysed by Gas Chromatography–Mass Spectrometry coupled with solid-phase micro-extraction (GC–MS–SPME) technique.

Moreover, the synergic combination of transglutaminase and sourdough fermentation has been evaluated in order to define their effects on the technological properties of the final product in conventional bread recipe. Rheological properties and crumb grain features were measured by Texture profile analysis (TPA), stress relaxation tests and Image analysis.

Finally, allergenic potential of the processed product was determined in order to evaluate the protein composition and its nutritional safety. Immunological characterization was conducted using and a specific antibody and sera from sensitive people.

2.2. Main Results

Results in this study demonstrated that microbial transglutaminase is able to explain its activity on protein substrate, of both gluten and gluten free flours. In particular, forming new protein aggregate at HMW by cross-linking reaction. In vitro,

via biochemical assay was determined that in wheat flour the prolamine are the main fraction involved in the formation of isopeptide covalent bounds. Furthermore, the addiction of sourdough determine a significant increase of the enzyme activity on flour protein substrate.

Sourdough fermentation, adopted in this study was able to increase the microbial metabolism and produce some interesting precursors of flavouring and leavening molecules.

Wheat sample bread obtained using transglutaminase and sourdough, results in a good quality in terms of texture properties, rheological characteristics, aroma profile, and longer shelf-life than control sample not processed.

Immunological analysis determine that the protein content of gluten-free dough, after biotechnological treatment is still safe for sensitive people.

That data constitute an important stride in food biotechnology and suggested the possibility to create innovative products with improved characteristics using new biological system.

3. Transglutaminase and Sourdough on Gluten-Free Dough

Abstract

The aim of this work was to evaluate the effects of transglutaminase and sourdough on gluten-free flours. Besides deamidation and incorporation of amines, transglutaminase catalyses protein cross-links, modifying dough structure. Sourdough from lactic acid bacteria and yeast modifies dough protein composition, determining proteolysis, which induce the formation of aroma precursor metabolites. The chemical-physical interactions of volatile molecules with various constituents of the matrix affect the retention of aroma compounds. Here, the effect on volatile

molecule profiles and on protein networks formation after transglutaminase treatment in sourdoughs obtained with four gluten-free flours belonging to cereals, pseudo-cereals and legumes (rice, corn, amaranth and lentil) was investigated. Sourdough was prepared with a two-step fermentation using *Lactobacillus sanfranciscensis* (LSCE1) and *Candida milleri* (PFL44), then transglutaminase was added after 21 h of fermentation at increasing levels. The results showed that transglutaminase had the capacity to modify gluten-free flour proteins and improve protein networks formation, involving mainly the prolamin protein fraction. This is particularly relevant for the production of gluten-free backed goods generally lacking of technological, structural and sensorial features compared with products obtained with wheat flour sourdough fermentation. Interestingly, transglutaminase treatment of sourdough affected also the volatile composition and indeed possibly the final organoleptic properties of the products.

3.1. Introduction

Transglutaminase (protein-glutamine γ-glutamyltransferase, E.C. 2.3.2.13) catalyses proteins cross-links through an acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutamine and the ɛ-amino group of peptide-bound lysine, resulting in the formation of inter- and/or intramolecular- ɛ-(γ-glutamyl) lysine isopeptide bonds. In the presence of primary amines, as aliphatic polyamines, the enzyme catalyses a transamidating reaction in which the primary amine group of polyamine, can replace lysine residues and results in the formation of N-mono (γ-glutamyl) polyamine. In the presence of a second reactive glutamine residue, the reaction may proceed to covalent cross-linking between two polypeptide chains via an N, N-bis (γ-glutamyl) polyamine bridge. In the absence of suitable

amines, water can act as a nucleophile causing deamidation of protein-bound glutamine residues (Kieliszek & Misiewicz, 2014; Lorand & Graham, 2003) The cross-linkages of protein, catalysed by transglutaminase are stable and causes various physical and chemical changes in food proteins, leading to an increased structure and texture of protein substrates. Transglutaminase has been successfully used in food processes to promote protein cross-linking, in particular the microbial enzyme, isolated from *Streptoverticillum mobaraense*. Transglutaminase was found to have a positive impact on the stability and volume of wheat dough as well as on the improvement of the baking quality of poor flour and, consequently on the texture of the bread (Marco & Rosselli, 2008). Gluten is the major factor involved in the structural properties of bakery products. Thanks to its ability to create aggregates, it gives dough viscosity, elasticity and cohesion, all features responsible for the baking performances (Delcour, Joye, Pareyt, Wilderjans, Btijs, & Lagrain, 2012). Gluten is composed of extensible, viscous gliadins and rigid, elastic glutenins. Moreover, transglutaminase-mediated deamidation of gliadins, is the main factor responsible for the development of celiac disease symptoms, but proteins belonging to gluten fraction are also responsible for other non-celiac gluten sensitivities. The only treatment for these people is lifelong adherence to a strict gluten-free diet (Dowd, Tamminen, Jung, Case, McEwad, & Beauchamp, 2014). This caused an increasing request for new products produced by using alternative cereals like rice, corn (Ferreira, 2016), sorghum (Trappey, Khouryieh, Aramouni, & Herald, 2015) and millet (Chhavi & Sarita, 2012), or pseudocereals such as buckwheat, amaranth (de la Barca, Rojas-Martìnez, Islas-Rubio, & Cabrera-Chavez, 2010) and quinoa (Brito, de Souza, Felex, Madruga, Yamashita, & Magnani, 2015). Flours of those plants do not contain gluten, the causative agent for celiac disease(Lamacchia, Camarca, Picascia,

Di Luccia, & Gianfrani, 2014). Moreover, pseudocereals are well known to be rich in proteins containing essential amino acids such as lysine (Alvarez-Jubete, Arendt, & Gallagher, 2009), limited in wheat and rye flours. On the other hand, the use of gluten-free flours is restricted due to the low texture and structure of their doughs, as well as the sensory quality of the baked products (Gallagher, Kunkel, Gormley, & Arendt, 2003). Nowadays, new consumer demands have emerged for food products with improved nutritional value or health benefit, posing new challenges also for the baking industry. The replacement of the gluten network in the development of gluten-free bakery products is a challenging task for the researchers of food science. The application of transglutaminase as an ingredient for gluten-free systems is a promising tool, promoting the formation of protein networks, thus affecting the microstructure of the dough.

In this study transglutaminase treatment was performed on sourdough obtained from gluten-free flours by a two-step fermentation of *L. sanfranciscensis* LSCE1 and *C. milleri* PFL44, 17 h and 4 h respectively. The use of lactic acid bacteria in the form of sourdough has been reported to have positive effects on wheat bread quality and staling (Clarke, Schober, & Arendt, 2002; Corsetti, et al., 2000; Crowley, Schober, Clarke, & Arendt, 2002). Sourdough is dough made of flour and water containing microorganisms including lactic acid bacteria and yeast in symbiotic combination. The sourdough applications in bread making are motivated by the beneficial effect on the flavor, texture, nutritional properties and extended shelf-life. These positive effects are associated with the metabolic activity of the selected pure cultures used in the composition of the sourdough, e.g. lactic acid fermentation, proteolysis, exopolysaccharide production and synthesis of volatile and antimicrobial compounds (Corsetti, Settanni, Chaves López, Felis, Mastrangelo, & Suzzi, 2007).

Due to the superior sensory quality and the prolonged shelf-life of the resulting baked goods, sourdough processes have retained their importance in modern baking technology (Stolz & Bocker, 1996; Vogelmann, Seitter, Singer, Brandt, & Hertel, 2009). For these reasons, the fermentation process of such alternative flours may improve both the sensory and baking qualities. Moreover, *L. sanfranciscensis* and *C. milleri* were selected on the basis of their ability to produce antimicrobial compounds, like medium chain fatty acids (MCFAs) and lactones, and interesting molecules in terms of organoleptic improvement of the product in scalar sourdough fermentation. This consortium was tested before by Vernocchi and collaborators (2008). In fact, the use of selected lactic acid bacteria and yeasts starter cultures is a prerequisite for ensuring a constant quality of sourdough bread (Vernocchi, Ndagijimana, Serrazanetti, Gianotti, Vallicelli, & Guerzoni, 2008). In addition, due to the increased demand of gluten-free products, the adaptability of various lactic acid bacteria and yeast starter strains in sourdoughs prepared from cereals or pseudocereals was investigated (Moroni, Arendt, Morrissey, & Dal Bello, 2010; Vogelmann, Seitter, Singer, Brandt, & Hertel, 2009).

More specifically, the effect on volatile compounds (VCs) and on protein network formation as a consequence of transglutaminase treatment added on sourdoughs derived from four gluten-free flours belonging to cereal (rice and corn), pseudo-cereals (amaranth) and legumes (lentil) was investigated. The sourdough was prepared with a two-step fermentation using *L. sanfrancisciensis* and *C. milleri* , then the microbial transglutaminase from *Streptoverticillum mobaraense* was added after 21 h of fermentation at increasing levels. Moreover, transglutaminase activity was also evaluated in relation to flour type and enzyme level.

In this context the overall aim of the research was to evaluate the potential of the combination of a previously set up microbial consortium and transglutaminase to improve the protein network formation and the volatile compound profiles of sourdough obtained from gluten-free flours such as, rice, corn, lentil and amaranth.

3.2. Materials and Methods

3.2.1. Raw Material and Chemicals

Four types of gluten-free flours, corn, rice, amaranth, and lentil, purchased from local markets, were used in the preparation of laboratory dough. All reagents and solvents (unless otherwise indicated) were of the highest purity and were obtained from Sigma-Aldrich (Milan, Italy).

3.2.2. Dough Preparation and Microbial Fermentation

In this study, dough batches were prepared by using tap water and four gluten-free flours. Sourdoughs were prepared according to the two-step fermentation process, based on straight interaction between lactic acid bacteria and yeast, in order to obtain combined metabolic activity. Previously, strain of lactic acid bacteria and yeast, both belonging to the culture collection of Department of Agricultural and Food Science (University of Bologna, IT), were selected (Vernocchi, Ndagijimana, Serrazanetti, Gianotti, Vallicelli, & Guerzoni, 2008). *Lactobacillus sanfranciscensis*, is considered the key organism for the acidification of sourdough and it is able to liberate aroma precursors (Gänzle, Vermeulen, & Vogel, 2007; Gobbetti & Corsetti, 1997) and *Candida milleri*, was chosen because characterized by the stable association with hetero-fermentative lactobacillus metabolism, as maltose-negative yeast (Gobbetti, 1998). Starters were cultivated separately on mMRS medium (Stolz,

Bocker, Hammes, & Vogel, 1995) at 32 °C for 48 h and Sabouraud Dextrose medium (Oxoid, Basingstoke, UK) at 27 °C for 48 h, respectively. Both strain cells, of *L. sanfranciscensis* (LSCE1) and *C. milleri* (PFL44), were harvested by centrifugation (4,000 × g, 15 min, at room temperature of 22 °C) until the late exponential phase of growth was reached and re-suspended in physiological saline solution (0.9% NaCl) at the cell density of 9.0 ± and 8.0 ± log cfu/ml, respectively. Four different sourdoughs, having dough yield (DY, dough weigh×100/flour weight) of 250, were prepared with the gluten-free flours. Initially, *L. sanfranciscensis*, was inoculated (7.0 log cfu/g) and the fermentation was carried out for 17h at 32 °C, then *C. milleri*, was added (6.0 log cfu/g) and the fermentation was continued for another 4 h, at 32 °C, for a total 21 h of fermentation. During all process the doughs were mixed and incubated in a temperature-controlled shaker, at 400 rpm. During the main three times of fermentation (time 0 , time 17 h, and time 21 h) the water activity (aw), measured by water activity meter (AquaLab Series 4TE from Decagon Device, Inc.) and pH values, determined by pH-meter (Crison Basic20) with a food penetration probe, were registered at room temperature. Contemporary cell concentration of *L. sanfranciscensis*, was analysed by plate counting on mMRS medium containing 0.1 g/L of cycloheximide after incubation at 30 °C for 48 h in anaerobiosis. Yeasts determined by plate counting on Sabouraud Dextrose medium (Oxoid, Basingstoke, UK) containing 0.2 g/L of chloramphenicol after incubation at 27 °C for 48 h. Rates of doughs and sourdoughs obtained were stored at −20 °C before the following chemical treatment and analyses.

3.2.3. Enzymatic Treatment with mTG

The microbial transglutaminase tested in this study, Activa WM transglutaminase from *S. mobaraense*, were purchased from Ajinomoto (specific activity: 100 U/g, Ajinomoto Foods Europe S.A.S., France). The Activa WM, enzymatic commercial powder, is composed of 1% transglutaminase and 99% maltodextrins. To induce protein cross-link in dough, the experiment was carried out by adding microbial transglutaminase on gluten-free flour dough. The treatment was performed using increasing levels of enzyme (0.5, 0.75, 1, and 2 U/g of flour) at 30 °C for 90 min. Also, sourdoughs obtained from several gluten-free flours were treated with increasing amount of microbial transglutaminase (0.5, and 1 U/g of flour) to evaluated the combined effect of sourdough fermentation and enzyme on protein profiles. More in detail, after the 21 h of fermentation, samples pH was evaluated and then samples were frozen in order to arrest the microbial activity. The cross-linking formation on protein substrates was checked by protein extraction and separation by SDS-PAGE (15%). The activity of microbial transglutaminase was evidenced by changes in protein pattern; the negative control has been performed without addition of enzyme.

3.2.4. Protein Extraction and Separation

The proteins were extracted with different buffer solutions in order to obtain total protein and enriched fraction extracts of protein classes like albumins/globulins (F1), prolamins (F2) and glutelins (F3). Total proteins were extracted under reducing conditions using the following buffer solution: 100 mM Tris–HCl pH 6.8, 4% (w/v) sodium dodecyl sulphate, 20% glycerol, 200 mM β-mercaptoethanol. Protein fractions were extracted following a sequential extraction protocol using various

solvents, according to the method of (Marco, Perez, Ribotta, & Rosselli, 2007), with minor modifications. In particular, the F1 was obtained by extraction with 5% sodium chloride solvent; it was then homogenised and centrifuged at 5500×g for 10 min at 4 °C. The F2 was then extracted by adding 50% 1-propanol to the flour residue, following the same procedure as in the F1 extraction. Finally, the F3 was extracted by adding 0.1 M NaOH containing 0.5% sodium dodecyl sulphate and 0.6% β-mercaptoethanol to the residue. Each step was repeated twice for better extraction and supernatants were collected. The protein content of the extract was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard and commercial Bradford solution (Bio- Rad Laboratories, Hercules, CA). The concentration was determined spectrophotometrically, at 595 nm using a microplate reader (VICTOR™ X3 Multilabel Plate Reader, PerkinElmer). Protein extracts were heated to 100 °C, for 5 min, and centrifuged for 3 min. Fifty micrograms of protein were separated by SDS-PAGE using a 15% acrylamide; gels were stained with 0.25% Coomassie Brilliant Blue R-250 in a methanol, water, and acetic acid (4:5:1 v/v) solution, at room temperature and were destained in the same solvent. The molecular weight values of the protein bands were estimated using Biomol BLUE plus prestained Protein Ladder (10–180 kDa), used according to the manufacturer's instructions. The protein stained bands were analysed using Aida scan software and the linear relationship between the stain intensity and the protein concentration was observed with each band. The relative grey level intensity of each protein bands analysed were quantified by Aida Image analyser v.4.14.

3.2.5. Biotin-labelled Cadaverine Incorporation Assay

In order to evaluate the ability of microbial transglutaminase to catalyse the incorporation of polyamines in gluten-free protein extracts, a microplate colorimetric assay was performed to check the incorporation of biotinylated cadaverine (BC) into glutaminyl residue of the gluten-free protein substrates, forming γ-glutamyl BC. The assay was carried out according to the method of (Lilley, Skill, Griffin, & Bonner, 1998) with some modifications. Microplate wells were coated with protein extracts (5 mg/mL), then BC and enzyme were added (5 mU). The assay was carried out under optimal condition of pH with 100 mM Tris HCl pH 8.0 buffer solution. The coated proteins were modified enzymatically due to incorporation of BC into glutamine residues. The incubation time for the microbial transglutaminase reaction was 2 h at 37 °C. The biotin-labelled products were detected by conjugation of extravidin–peroxidase, according to Lilley and collaborators (1998) with 10 mM cystamine as a transglutaminase inhibitor replacing EDTA in negative controls. The microbial transglutaminase activity was expressed as a change in absorbance per unit time (ΔA450/h) per mg of enzyme used (U/mg).

3.2.6. SPME-GC-MS Analysis

VCs were analysed by GC–MS–SPME technique. For each analysis, 3 g. of samples were placed in 10 mL sterilised vials, sealed by PTFE/silicon septa, adding 10 µL of 4-methyl-2-pentanol (final concentration of 33 mg/L) as internal standard, and pre-equilibrated for 10 min at 45 °C. Afterwards, a fused silica fibre covered by Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS StableFlex, Supelco, Steiheim, Germany) was introduced and maintained in the sample head-space for 40 min. Then, it was removed and immediately inserted into GC-MS

injector for desorption of compounds, at 250 °C for 10 min. For peak detection, an Agilent Hewlett Packard 6890 gas chromatograph equipped with a mass spectrometer detector 5970 MSD (Hewlett Packard, Geneva, Switzerland) and a Varian (50 m × 320 mm × 1.2 μm) fused silica capillary column was used. The conditions used were as follows: injection temperature, 250 °C; detector temperature, 250 °C; carrier gas (He) flow rate, 1 ml/min. The oven temperature was programmed as follows: 50 °C for 1 min; from 50 °C to 65 °C at 4.5 °C/min; from 65 °C to 230 °C at 10 °C/min; the final temperature was maintained for 25 min. The volatile compounds were identified by matching the retention times with those of pure compounds (Sigma-Aldrich, Milan, Italy). Identities were confirmed by searching mass spectra in the available databases (NIST, version 2005; Wiley, version 1996). All the GC–MS raw files were converted to netCDF format via Chemstation (Agilent Technologies) and subsequently processed with the XCMS toolbox (http://metlin.scripps.edu/download/). XCMS software allows automatic and simultaneous retention times alignment, matched filtration, peak detection and peak matching. The resulting table containing information such as peak index (retention time-m/z pair) and normalised peak area was exported into R (http://www.r-project.org) (Serrazanetti, Ndagijimana, Sado-Kamdem, Corsetti, Vogel, Ehrmann, et al., 2011). Volatile compounds were expressed as percentage peak area. Only identified compounds which reached a concentration more than 0.1% of the total peak area in a sample was used for subsequent statistical analysis. Volatile compounds were determined on sourdoughs of four gluten-free flours during all fermentation process (t0, t17, and t21) and on sourdough treated with 0.5 U of microbial transglutaminase/g of flour for 90 min at 30 °C, prepared as described in

paragraph "Enzymatic treatment with mTG". A formulation without any addition of enzyme was used as control.

3.2.7. Statistical Analysis

The data reported are the means of three repetitions and are expressed as mean ± standard deviation (SD). Data were compared using one- or two-way analysis of variance (ANOVA), with a significant difference ($P < 0.05$) of at least 95%, with Bonferroni post-test, using GraphPad Prism software. The percentage concentrations of the molecules, detected by GC–MS–SPME analysis, were used for principal component analysis (PCA) (Vannini, Ndagijimana, Saracino, Vernocchi, Corsetti, Vallicelli, et al., 2007).

3.3. Results and Discussion

3.3.1. Fermentation Parameters and Microbial Cell Counts

Sourdoughs, made with rice, corn, lentil, and amaranth flours, were monitored in terms of lactic acid bacteria and yeasts concentration during the fermentation process (t0, t17, and t21). The fermentation features of the gluten-free sourdough are summarised in Table 3.1.

Flour	t_0				t_{17}				t_{21}			
	pH	aw	Cell counts (log cfu/g)		pH	aw	Cell counts (log cfu/g)		pH	aw	Cell counts (log cfu/g)	
			LAB	Yeast			LAB	Yeast			LAB	Yeast
R	6.12	1.004	7.18	<1	3.50	0.990	8.47	3.40	3.43	1.000	9.39	7.50
C	5.58	0.997	6.90	<1	3.43	0.993	8.99	2.67	3.38	0.998	8.82	6.90
L	6.30	0.988	7.27	<1	4.92	0.988	9.06	2.38	4.56	0.988	9.04	7.03
A	6.42	0.988	6.94	<1	4.46	0.982	9.02	3.20	4.07	0.985	9.30	6.85

Table 3.1. Fermentation features of the gluten-free sourdough obtained using *L. sanfranciscensis* and *C. millleri*, at three time of fermentation (t0, t17, and t21). R: rice, C: corn, L: lentil, A: amaranth

After 21 h of fermentation lactic acid bacteria reached levels between 9.39 and 8.82 log cfu/g and the yeast between 7.50 and 6.85 log cfu/g. It is evident that the ratio between yeast and lactic acid bacteria was about 1:100 that is considered on the

basis of wide literature typical of stable wheat sourdough (De Vuyst & Neysen, 2005). By contrary, in a gluten-free sourdough based on corn starch, rice and pea proteins, a lower ratio between lactic acid bacteria and yeast was obtained, possibly due to the higher yeast cell load at the end of fermentation (Picozzi, Mariotti, Cappa, Tedesco, Vigentini, Foschino, et al., 2016). In order to evaluate the acidification effect of lactic acid bacteria during the fermentation process, sourdough pHs were monitored. At the initial time (t0), pHs of the different sourdoughs were similar and ranged from 5.6 to 6.4. During the first step of fermentation, after *L. sanfranciscensis* addition and 17 h of fermentation (t17), a high acidification of sourdoughs was observed, with pH values ranging between 3.4 and 4.9. The acidification was greater in rice and corn matrices with 3.5 and 3.4 values. lentil and amaranth showed 4.9 and 4.5 pH values, respectively. Finally, after 21 h of fermentation (t21), pH analysis showed a slight acidification effect on sourdoughs, with pH values between 3.4 and 4.6. The main effect of lactobacillus species in the sourdough fermentation process is the rapid acidification of the matrix. This process is caused by the fast conversion of fermentable carbohydrates into mainly lactic acid, but also other organic acids such as acetic acid, formic acid, and ethanol. At pH below 4.0, there is a sizable effect of protein solubilisation, which influences directly the proteolysis process and also the interaction of proteins with other molecular partners. As food products are composed of a wide range of ingredients such as proteins and carbohydrate-based polysaccharides, often interaction between protein and polysaccharides occurs. Complex formation between proteins and polysaccharides occurs at pH values below the isoelectric point of the proteins and at low ionic strength. Protein molecules have a net positive charge and behave as polycations at pH values below the isoelectric point (de Kruif, Weinbreck, & de Vries, 2004). At mildly acidic and neutral pH

values, which are typical of most foods, carboxyl containing polysaccharides behave as polyanions. Electrostatic complex formation between proteins and anionic polysaccharides generally occurs in the pH range between the pK value of the anionic groups (carboxyl groups) on the polysaccharide and the isoelectric point of the protein (Ye, 2008). Some consequences of acidification and proteolysis include the accumulation of amino acids, flavour precursors, and the change in dough rheology and texture, resulting in a large reduction of elasticity and firmness of the dough (Arendt, Ryan, & Dal Bello, 2007).

3.3.2. Effect of Sourdough Fermentation on VCs

The generation of volatile compounds occurred in sourdoughs mainly because of microbial metabolism, enzymatic oxidation or autoxidation of flour lipids; however, some compounds were already present in raw samples and are possibly responsible of the characteristic taste of the gluten-free flours (Figure 3.1 A, and B). To describe volatile compound profiles of sourdoughs obtained with gluten-free flours, during the three times of the fermentation process (t0, t17, and t21), the molecules were detected by GC–MS–SPME technique.
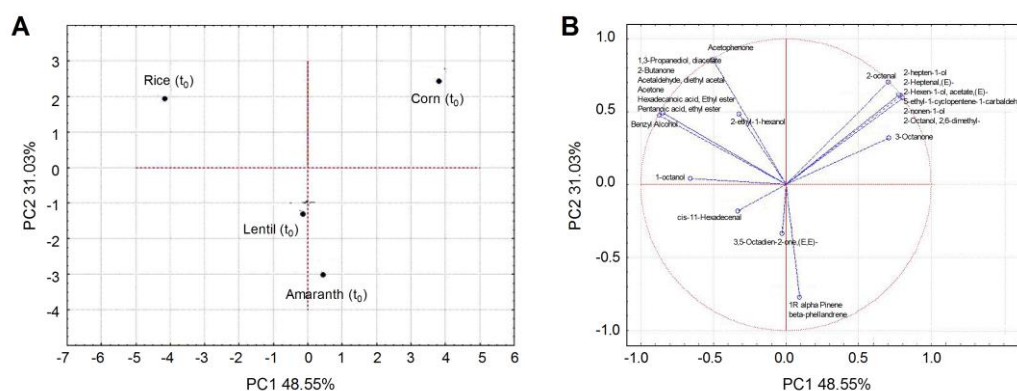
Figure 3.1. PCA of VCs present in gluten-free doughs before the fermentation process (t0). A: PCA score plot on the two first factors, PC1 (48.55%) and PC2 (31.03%); B: loading plot of the VCs selected on the first two factors obtained from the PCA.

Molecules belonging to different chemical classes, alcohols, aldehydes, esters, ketons, MCFAs and terpenes, were identified. Specific volatile fingerprints, in relation to the different flours used in preparation of sourdoughs, were recorded during all three principal times of fermentation (t0, t17, and t21). Figure 3.2 shows the principal classes of components, which characterise the metabolite activity of the selected microbial consortium, in the studied flours, during the fermentation process.
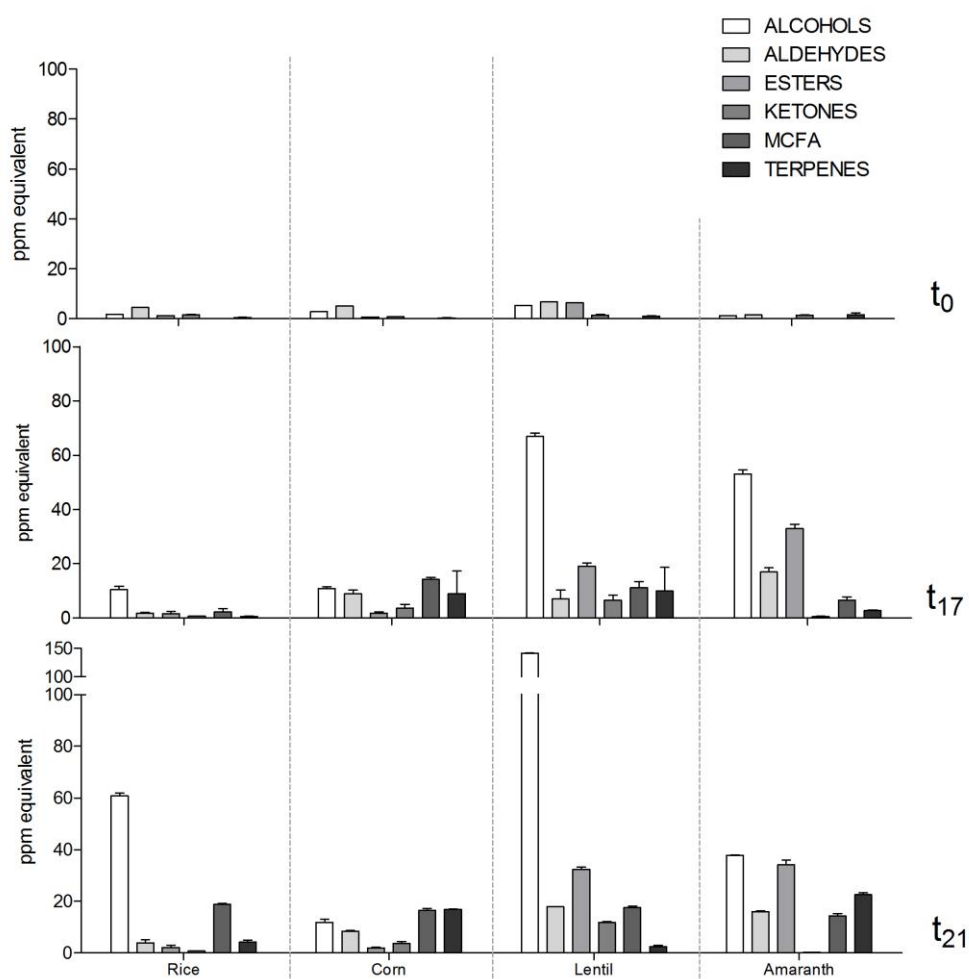
Figure 3.2. VCs profiles of gluten-free flour sourdoughs, analysed by GC–MS SPME, during three different times of fermentation: at the beginning (t0), after 17 h (t17) and after 21 h (t21). VCs are reported grouped in main metabolite classes: alcohols, aldehydes, esters, ketones, MCFAs, and terpenes.

The generation of volatile compounds occurred in sourdoughs mainly because of microbial metabolism (e.g. alcohols, 2,3-butanedione, esters and acids) and

enzymatic oxidation or autoxidation of flour lipids (e.g. aldehydes, ketones and 2-pentylfuran). During the first step of fermentation (t17), *L. sanfranciscensis* contributed differently to the synthesis of aroma compounds in relation to the flour considered. In fact, in lentil sourdough, a significant increase of alcohols, esters (mainly ethyl acetate) and ketones was observed after 17 h of fermentation, thus ascribable to lactic acid bacteria metabolism. Concerning amaranth, the fermentation of *L. sanfranciscensis* mainly induced an increase of alcohols, aldehydes, ester and terpenes, while keton levels remained the same. In corn sourdough only esters increased after the 17 h of fermentation by *L. sanfranciscensis*, mainly due to the concentration of ethyl acetate. Interesting is the production of hexanoic acid in rice after 17 h of fermentation by *L. sanfranciscensis*. This MCFA was also produced in A sourdough after *C. milleri* inoculum. After 21 h the volatile compound profiles reflected the activity of lactic acid bacteria and yeast. To better pinpoint the differences in relation to the fermentation steps (t0, t17, and t21) and to the microbial transglutaminase supplementation at 21 h, a principal component analysis of GC–MS–SPME data was performed for each flour type. The two-step fermentation process significantly affected the projection of samples on the factorial plane independently on the flour type. The fermentation process drastically affected volatile compound composition of the gluten-free flours and the first step of fermentation resulted in samples well separated on the basis of the PC2 (>17% of the total variance), from the dough before fermentation (t0), indicating that *L. sanfranciscensis* contributed in a significant way to the complexity of the aroma profile. More significant was the contribution of *C. milleri*. Since the samples at t21 resulted separated from the dough before fermentation (t0) and samples at t17 both on PC1 (>60%) and PC2. Representative

PCA and its score plot are reported in Figure 3.3 for corn and lentil sourdoughs, while the complete list of volatile compounds is reported in Table 3.2.
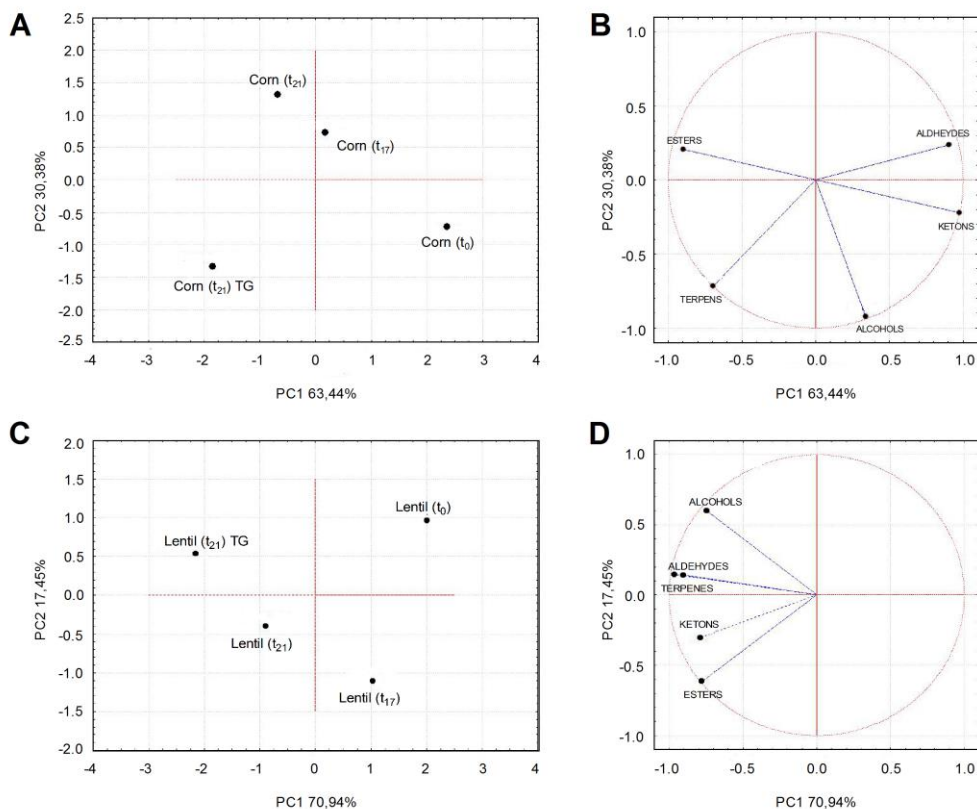
Figure 3.3. PCA of VCs of principal metabolite classes present in corn and lentil sourdoughs, based on the molecules concentration at three times of fermentation (t0, t17, and t21) and t21 treated with TG (0.5 U/g of flour) for 90 min at 30 °C. A: Corn sourdough: PCA score plot on the two first factors, PC1 (63.44%) and PC2 (30.38%) and B: loading plot of the VCs selected on the first two factors obtained from the PCA. C: Lentil sourdough: PCA score plot on the two first factors, PC1 (70.94%) and PC2 (17.45%) and D: loading plot of the VCs selected on the first two factors obtained from the PCA.

The addition of microbial transglutaminase contributed to the separation of samples on the factorial plane only for lentil and corn sourdough (Figure 3.3). Only in these sourdoughs, the supplementation of microbial transglutaminase induced a significant modification of the aroma profile resulting in a separation of the samples recorded after 21 h only on the PC2 able to explained the 30.38% and the 17.45% of the total variance in corn (Figure 3.3 A, and B) and lentil (Figure 3.3 C, and D), respectively.

	RICE				CORN				LENTIL				AMARANTH			
	t0	t17	t21	t21 TG	t0	t17	t21	t21 TG	t0	t17	t21	t21 TG	t0	t17	t21	t21 TG
ALCOHOLS	**3.40**	**3.50**	**0.80**	**1.60**	**11.60**	**4.80**	**4.50**	**6.90**	**4.70**	**9.90**	**16.10**	**18.40**	**0.00**	**15.80**	**7.60**	**8.40**
1-octanol	0.70	0.60	0.00	0.00	0.00	0.00	0.10	0.20	0.90	0.50	0.00	2.60	0.00	0.30	0.00	0.00
2,4-decadien-1-ol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.10	0.00	14.70	7.50	8.40
2-ethyl-1-hexanol	1.40	1.80	0.00	0.20	1.00	0.00	0.00	0.00	1.70	0.60	0.00	0.00	0.00	0.30	0.00	0.00
2-hepten-1-ol	0.00	0.10	0.30	0.00	0.60	1.20	1.70	2.10	0.00	0.60	1.40	1.40	0.00	0.20	0.00	0.00
2-Hexen-1-ol(E)-	0.00	0.00	0.00	0.00	3.60	0.60	0.00	0.00	1.20	7.70	13.40	12.90	0.00	0.00	3.80	3.90
2-nonen-1-ol	0.00	0.00	0.50	0.80	0.40	1.60	1.50	1.20	0.00	0.80	1.30	1.30	0.00	0.00	0.00	0.00
2-Octanol, 2,6-dimethyl-	0.00	0.00	0.00	0.60	6.00	0.20	0.60	1.00	0.80	0.00	0.00	0.10	0.00	0.10	0.10	0.00
Benzyl Alcohol	1.30	1.00	0.00	0.60	1.20	1.20	0.60	2.40	0.10	0.20	0.00	0.00	0.30	0.30	0.00	0.00
ALDEHYDES	**2.40**	**2.90**	**5.80**	**14.30**	**24.60**	**16.70**	**19.50**	**11.60**	**3.90**	**0.90**	**29.00**	**27.80**	**0.00**	**2.60**	**4.90**	**5.40**
2-Heptenal,(E)-	0.00	0.00	5.60	13.20	3.60	10.50	12.20	6.60	0.00	0.00	28.90	27.00	0.00	0.00	0.50	0.70
2-octenal	1.30	0.00	0.00	0.00	9.80	0.00	2.50	2.30	0.60	0.50	0.10	0.80	0.00	0.00	0.60	0.80
5-ethyl-1-cyclopentene-1-carbaldehyde	0.00	0.00	0.00	0.00	11.20	2.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9,17-Octadecadienal, (Z)-	0.00	0.00	0.00	4.00	0.00	3.70	4.60	2.70	0.00	0.00	0.00	0.00	0.00	2.20	3.80	3.90
cis-11-Hexadecenal	1.10	2.90	0.20	1.10	0.00	0.00	0.20	0.00	3.30	0.40	0.00	0.00	0.00	0.40	0.00	0.00
ESTERS	**9.50**	**4.00**	**6.70**	**6.80**	**0.00**	**3.60**	**9.40**	**8.70**	**0.00**	**13.20**	**16.90**	**15.50**	**0.00**	**26.30**	**29.70**	**30.70**
1,3-Propanediol, diacetate	8.10	2.50	2.00	0.00	0.00	0.00	2.20	3.30	0.00	0.10	0.30	0.80	0.00	0.80	0.90	0.80
14-Octadecenoic acid, methyl ester	0.00	0.00	0.00	0.70	0.00	1.50	1.70	1.00	0.00	0.00	0.00	0.00	0.00	0.80	2.10	2.20
2-Hexen-1-ol, acetate,(E)-	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.10	0.00	0.30	0.80	0.70	0.00	0.10	0.00	0.00
Acetaldehyde, diethyl acetal	0.60	1.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl Acetate	0.00	0.00	2.90	4.00	0.00	1.20	2.40	2.10	0.00	12.80	15.80	14.80	0.00	23.70	25.90	27.00
Hexadecanoic acid, Ethyl ester	0.70	0.00	1.70	1.90	0.00	0.90	2.90	1.90	0.00	0.00	0.00	0.00	0.00	0.70	0.80	0.40
Pentanoic acid, ethyl ester	0.10	0.30	0.10	0.20	0.00	0.00	0.10	0.30	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.30
KETONES	**4.80**	**1.80**	**1.90**	**2.70**	**1.70**	**0.90**	**0.50**	**0.50**	**1.10**	**2.50**	**1.90**	**3.10**	**0.00**	**0.00**	**3.50**	**3.10**
2-Butanone	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.70	0.00	0.00	2.80	2.30
3,5-Octadien-2-one	0.00	0.30	0.50	1.00	0.00	0.20	0.10	0.30	0.30	1.10	0.30	0.40	0.00	0.50	0.50	0.80
3-Octanone	0.00	0.00	0.00	0.20	0.60	0.10	0.40	0.00	0.50	0.30	0.80	1.50	0.00	0.00	0.00	0.00
Acetone	2.00	0.00	0.30	0.30	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acetophenone	2.10	1.50	1.10	1.20	1.10	0.60	0.00	0.10	0.30	1.10	0.30	0.50	0.00	0.00	0.20	0.00
MCFA	**0.00**	**0.30**	**1.80**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**1.50**	**1.30**
Hexanoic acid	0.00	0.30	1.80	0.00	0.00	0.50	0.30	0.30	0.00	5.90	0.20	0.00	0.00	1.50	1.50	1.30
TERPENES	**1.70**	**0.70**	**0.20**	**0.40**	**1.80**	**0.50**	**0.30**	**0.30**	**1.70**	**5.90**	**0.20**	**0.20**	**1.50**	**1.40**	**1.20**	**1.10**
1R-a-Pinene	1.70	0.70	0.20	0.40	1.80	0.50	0.30	0.30	1.10	5.90	0.20	0.20	1.50	1.30	1.20	1.10
beta-Phellandrene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.00	0.00	0.00	0.50	0.10	0.00	0.00

Table 3.2. Significant (p<0.05) VCs, expressed as percentage of peak area, determined in gluten-free sourdoughs at the three time of fermentation (t0, t17 and t21) and at t21 after TG treatment (0.5 U/g of flour). Only identified VCs which reached a concentration of 0.1%, at least in a sample, were reported. The coefficients of variability were lower than 4%.

3.3.3. mTG-mediated BC Incorporation on Different Protein

The transamidase activity of microbial transglutaminase was checked on the four gluten-free flours both on total extracts and on enriched fractions by BC incorporation assay. The objective of this analysis was to evaluate and compare the presence of transglutaminase's substrate proteins on gluten-free flour extracts. Results on total protein extracts (Figure 3.4, insert), showed that proteins from gluten-free flours reached high level of BC incorporation. In fact, rice, corn, and amaranth substrates showed incorporation values in the range 50–60 U/mg of enzyme; whereas for lentil substrates the value of 17.02 ± 2.27 U/mg of enzyme was definitely lower when compared to the others. Then, microbial transglutaminase activity, detected by BC incorporation, was analysed on different protein-enriched fractions: the albumin/globulin (F1), prolamin (F2) and glutelin (F3) fractions. The aim of using these different protein fractions was to provide information about the kind of proteins mainly involved as substrates for the transamidase activity of microbial transglutaminase. The use of protein-enriched fractions significantly increased the activity of the enzyme, probably because the protein substrate resulted more exposed to the action of microbial transglutaminase (Figure 3.4).

Results showed a clear increase in the incorporation of BC, mainly in the F2 for all the different types of flours. In particular, F1 from lentil flours showed a specific activity of 212.9 ± 2.5 U/mg of enzyme and this value was not significantly different for A (187 ± 1.7 U/mg of enzyme), while it was undetectable for rice and C. F3 resulted in the most variable fraction in terms of enzyme activity. In general, F2 showed the highest enzyme activity with no significant differences among gluten-free flours, excepted lentil flour that showed the highest enzyme activity (849.1 ± 54.9 U/mg of enzyme) for this protein fraction.

To explain the differences in terms of amount of BC incorporation among total extracts and single fractions (F1, F2, and F3), it is possible to hypothesise in the four gluten-free total extracts, and in particular in lentil, the presence of some inhibitors of the microbial transglutaminase BC incorporation activity. Among these, free polyamines, as microbial transglutaminase substrate could compete with BC and indeed could be responsible for the lower activity checked in total extracts when compared to single fractions (F1, F2, and F3). About this, it is know that, among foods, legumes have a high polyamines content explaining the value of about 30% when compared to other gluten-free total protein extracts.
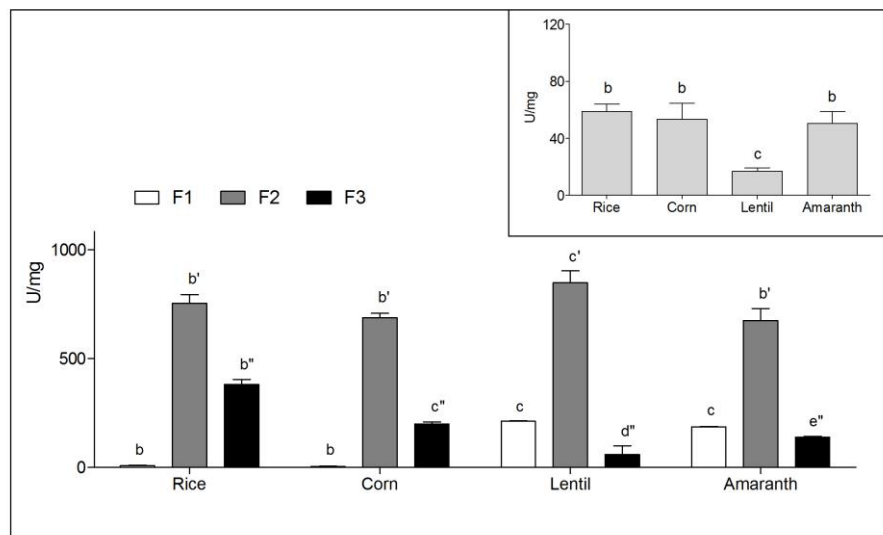
Figure 3.4. Transamidase activity of microbial transglutaminase on total protein extracts (insert) and enriched protein fractions of albumins/ globulins (F1), prolamins (F2) and glutelins (F3) of gluten-free flours. Specific activity was expressed as U/mg; values are mean ± SD of three replicates. Means of samples were compared by twoway ANOVA with a significant difference ($P < 0.05$) of at least 95%, with Bonferroni post-test. Samples labelled by the same letter are not significantly different.

As the procedure of extraction of the three protein fractions removed small molecules, among which free polyamines that are possibly extracted within the F1 fraction, this fraction resulted the lowest if compared with F2 and F3. Finally, as described in material and method section, total protein and enriched fractions extracts were obtained by the sequential extraction procedure with different buffers.

By comparing the four gluten-free flours, results showed that there was a similar trend between the microbial transglutaminase activity on the total protein and the F3 fraction, probably due to a similar composition of the buffers used to extract them. In fact, both the total protein- and the F3- extracting buffers contained sodium dodecyl sulphate and β-mercaptoethanol, which can deeply affect conformation and charge of proteins. These results show that microbial transglutaminase is active on gluten-free flours protein substrates, especially on F2 fraction. The highest signal of BC incorporation depends on the better capacity of microbial transglutaminase to catalyse transamidase reaction, and is probably influenced by the high content of glutamine residues present in F2. In fact, the acyl-transfer reaction catalysed by microbial transglutaminase is based on the formation of covalent bonds between glutamine and primary amine residues in proteins (De Jong & Koppelman, 2002).

3.3.4. mTG-mediated Cross-Linking

To investigate the protein cross-links formation in gluten-free flours after microbial transglutaminase treatment, total protein extracts from gluten-free flours doughs and sourdoughs were separated by SDS-PAGE. The effect of microbial transglutaminase, at increasing levels (0, 0.5, 0.75, 1, and 2 U/g of flour) caused the formation of protein aggregates, with increased molecular weight, both in dough and in sourdoughs, at a comparable level. Even though in sourdough the low pH was not the Activa WM TG optimum, in agreement with the manufacture product datasheet, it showed protein aggregation effects. These aggregates were identified in the wells, as well as at the top of the resolving gel. The enzyme effects on dough and sourdough proteins were observable as the disappearance of numerous protein bands in SDS-PAGE (Figure 3.5, grey lanes), possibly as a consequence of the formation of

large protein polymers (Figure 3.5, black lanes). The microbial transglutaminase addiction was visible as a clear band at around 35 kDa. The intensities of SDS-PAGE stained protein bands were analysed by Aida Image analyser and, after quantification, transglutaminase-treated and -untreated samples were compared.
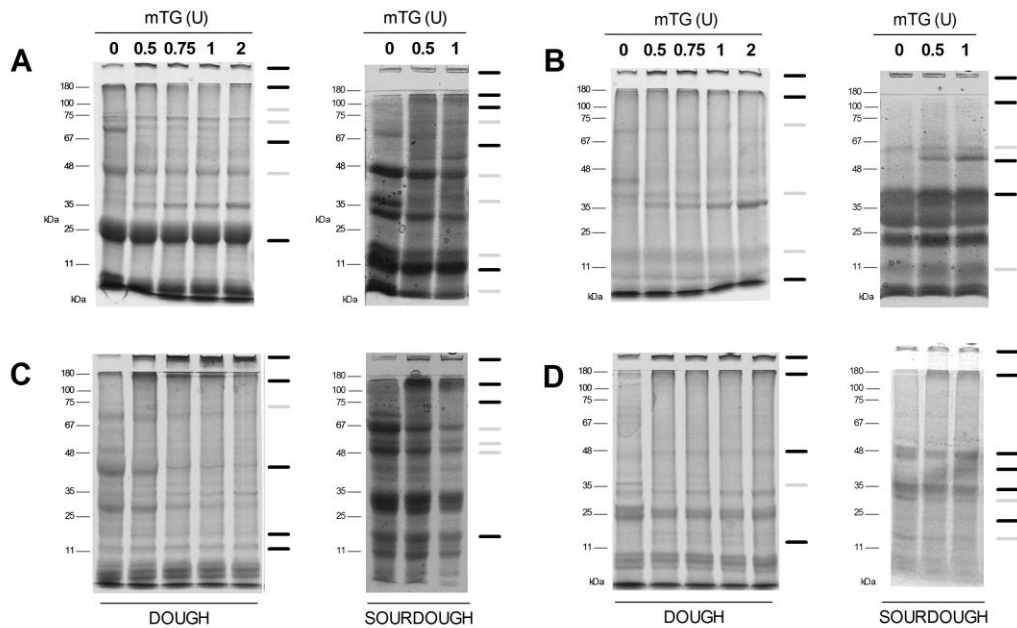
Figure 3.5. Cross-linking of protein substrates from gluten-free dough and sourdough treated with increasing concentrations of microbial transglutaminase (0; 0.5; 0.75; 1 and 2 U/g of flour) for 90 min at 30 °C. a Rice; b corn; c lentil and d amaranth. SDS-PAGE (15%) stained with Coomassie Brilliant Blue R-250; black and grey hyphens indicate bands whose intensity increased or decreased, respectively, by increasing amount of enzyme.

The reticulating effect of microbial transglutaminase in rice dough (Figure 3.5 A) caused the formation of protein aggregates, especially in the resolving–stacking boundary region and in the gel region of 20–40 kDa MW. Contrarily, a clear decrease of protein bands occurred in the region around 75–40 kDa, according to the level of enzyme supplied. In proteins derived from rice sourdough, the aggregation effect of microbial transglutaminase involved mainly LMW proteins at 11 kDa, whereas, the disappearance of the bands was evident in the whole resolving area of the gel. Microbial transglutaminase treatment on corn dough (Figure 3.5 B) caused protein aggregates mainly in resolving-stacking boundary region, effect that was enzyme

dose-dependent. In corn sourdough treated with microbial transglutaminase, the formation of protein aggregates was greater in the region of 67–11 kDa. Corn sourdough protein pattern was characterised by an increased amount of protein at LMW (below 25 kDa), that were absent in not fermented dough. Effects of microbial transglutaminase treatment on lentil dough are reported in Figure 3.5 C, which evidently showed the formation of protein aggregates at HMW, already at low enzyme dosage (0.5 U/g of protein). The increase of aggregates was parallel to a decrease of protein bands in region around 70 kDa, where a strong decrease occurred by increasing the enzyme amount. Also an increase of proteins in the range 11–17 kDa took place in presence of microbial transglutaminase. In sourdough, the enzyme effect was observed also at LMW region with an increase of proteins below 25 kDa, that were absent in not fermented dough. In the same manner, microbial transglutaminase treatment on amaranth dough (Figure 3.5 D), caused the formation of protein aggregates at HMW in the resolving-stacking boundary region. Moreover, here was an increase of protein bands amount in region 35–11 kDa. The increase in the HMW region was counterpoised to a decrease in the region around 30 kDa, where a clear decrease occurred with the increase of enzyme amount. In sourdough, the treatment with enzyme resulted mainly in protein aggregates at the top of the resolving gel. All sample doughs treated with microbial transglutaminase, with and without fermentation, resulted in protein pattern changes. In particular, the major effect was the formation of protein aggregates at HMW region of the gel, according to the increasing amount of enzyme. Proteins from lentil sourdough resulted as the best gluten-free protein substrate in the performed study conditions. One of the effects of the fermentation process on the gluten-free dough protein pattern, was the proteolysis that give rise to smaller polypeptides or amino acids (Di Cagno, et al.,

2002) (Gottfried & Werner, 1988). The proteolytic activity of lactic acid bacteria together with the acidification, strongly influence the physiochemical characteristics of the dough, as a consequence of protein changes (Arendt, Ryan, & Dal Bello, 2007). The cross-linking effect of microbial transglutaminase increases the formation of protein network, which may affect the protein structure of dough made with gluten-free flours and also after the fermentation process. lactic acid bacteria and microbial transglutaminase may affect protein structure of dough also by altering protein solubility, which depends on the hydrophilicity and hydrophobicity of the surface of the molecule that enters into contact with surrounding water. Intrinsic physico-chemical characteristics of the molecule and extrinsic factors of the solution, such as pH, deamidation of glutamine residues, and organic solvents, influence the solubility of proteins, all parameters that were studied in the present work and that were shown to be affected by sourdough fermentation in combination with microbial transglutaminase (Renzetti, Behr, Vogel, & Arendt, 2008). Moreover, affecting water activity (aw), probably by altering the interaction among proteins and water molecules, microbial transglutaminase may influence the final water content in dough matrix (Renzetti, Behr, Vogel, & Arendt, 2008). In fact, microbial transglutaminase increases the number of inter- and intra-chain peptide cross-links and increases as consequence protein–water interactions. Hence, both the deamidation reaction and the formation of cross-links catalysed by microbial transglutaminase directly influence aw in different protein substrates.

3.4. Conclusions

Gluten proteins play an important role in structure-building during all the bread bakery process, starting from dough formulation. These protein networks are

essential not only for leavening but also to retain gas and volatile metabolites produced during yeast fermentation. Cereal gluten proteins are, however, also involved in celiac disease and in other non-celiac gluten sensitivities. In view of this, gluten-free flours on one hand present the disadvantage to be hardly usable in bakery products; on the other hand, they represent the only solution to prevent the occurrence of celiac disease symptoms. The aim of our study was to obtain gluten-free doughs enhanced in protein aggregation, structural stability and volatile compounds profiles. Results showed that the properties of proteins from gluten-free flours, in terms of dough protein structure, could be successfully improved by the action of microbial transglutaminase that promoted the formation of protein networks. Moreover, the combination of microbial transglutaminase with sourdough, checked for the first time in gluten-free flours in the present paper, was changed when microbial transglutaminase and sourdough actions were combined: in detail, these two factors enhanced the formation and accumulation of volatile compounds that are interesting for leavening, aroma and also preservation of bakery products. The protein network formation via microbial transglutaminase occurred in all the gluten-free flour proteins, mainly in the prolamin-enriched fraction, being those from lentil sourdough the best reticulated proteins. In lentil, this combination caused the formation of volatile compounds belonging to terpenes, alcohols and aldehydes; all precursor metabolites of aroma compounds. This may affect the organoleptic properties of the final product. Further studies are required to fully understand the modifications brought by sourdough and microbial transglutaminase on gluten-free flour proteins; however, these data highlight the useful combination of sourdough and microbial transglutaminase for dough improvement.

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4. Transglutaminase and Sourdough on Wheat Baked Product

This chapter is based on:

Abstract

The combined use of the protein reticulating enzyme transglutaminase (TG) and of a selected microbial consortium of *Lactobacillus sanfranciscensis* and *Candida milleri* for improving the rheological properties, aroma, and shelf-life of a bakery product was evaluated. A microbial transglutaminase, showing the highest activity over a wide temperature range on different protein substrates, was selected among different types. Results showed that this transglutaminase was able to produce isodipeptide bonds, especially in the gluten fraction, leading to the

formation of protein aggregates, which improved the structure of a sourdough bakery product. The microbial transglutaminase in combination with sourdough exhibited a positive synergistic effect allowing the production of flavor-enriched bread, with rheological properties similar to those of standard bread.

4.1. Introduction

Bread is a fundamental food in the Western world and it is generally viewed as a perishable commodity, due to its fast decrease of freshness features and its rapid staling (Minervini, De Angelis, Di Cagno, & Gobbetti, 2014). Lactic acid bacteria and yeasts in the form of sourdough have been reported to have positive effects on wheat bread quality and staling (Clarke, Schober, & Arendt, 2002; Corsetti, et al., 2000; Crowley, Schober, Clarke, & Arendt, 2002) as they are responsible for the capacity of dough to leaven, while acidifying it (De Vuyst & Neysen, 2005). Traditional sourdough obtained with selected microorganisms is able to increase bread shelf-life by delaying staling (R. S.; Chavan & Chavan, 2011) and improve bread properties through enhancing its nutritional value, taste, and aroma profile (Arendt, Ryan, & Dal Bello, 2007; Hansen & Schieberle, 2005; Poutanen, Flander, & Katina, 2009). However, the use of lactic acid bacteria may affect the rheology of leavened bakery products through a strain-dependent proteolytic activity (Gobbetti, Smacchi, & Corsetti, 1996). These rheological properties, besides gas retention, depend on gluten proteins, composed of extensible, viscous gliadins and rigid, elastic glutenins. Chemical agents or cross-linking enzymes, such as glucose oxidase, peroxidase, or transglutaminase (TG), have been reported to improve dough handling properties and to increase fermentation stability, and loaf volume (Caballero, Gómez, & Rosell, 2007; Steffolani, Ribotta, Perez, & Leon, 2010). In

particular, transglutaminase (EC 2.3.2.13) is an important enzyme for the food industry (Basman, Koksel, & Ng, 2002) as it catalyses the formation of protein cross-links resulting in extensive nets (Nonaka, et al., 1989). The formation of protein polymers, as a result of transglutaminase activity, can modify the rheological properties of gluten (Köksel, Sivri, Ng, & Steffe, 2001) and allow the transformation of a very weak gluten into a very strong one (Larre, Denery-Papini, Popineau, Deshayes, Desserme, & Lefebvre, 2000). In previous studies, the positive effects of transglutaminase application on wheat-based baked products have been described (Renzetti, Behr, Vogel, & Arendt, 2008), (Gerrard, Newberry, Ross, Wilson, Fayle, & Kavale, 2000). The effects of transglutaminase on empirical rheological properties of dough (Basman, Koksel, & Ng, 2002; Marco, Perez, Ribotta, & Rosselli, 2007) and on the formulation of sourdough (Arendt, Ryan, & Dal Bello, 2007; Clarke, Schober, & Arendt, 2002) in order to obtain good-quality bread have been described. To date, however, the combined use of the two biological agents, i.e., transglutaminase and sourdough, has never been reported.

In the present work, we tested the possibility of improving bread quality through the combined use of sourdough and a protein-reticulating enzyme. transglutaminase was selected in order to improve rheology (Gerrard, Fayle, Wilson, Newberry, Ross, & Kavale, 1998), while sourdough based on *Lactobacillus sanfranciscensis* and *Candida milleri* was chosen for its ability to improve the aroma profiles and extend the shelf-life of the final product (Scarnato, Serrazanetti, Aloisi, Montanari, Lanciotti, & Del Duca, 2016; Vernocchi, Ndagijimana, Serrazanetti, Gianotti, Vallicelli, & Guerzoni, 2008). *L. sanfranciscensis* is a key organism for sourdough acidification and produces aroma precursors (Gänzle, Vermeulen, & Vogel, 2007; Gobbetti, Smacchi, & Corsetti, 1996), while *C. milleri* is able to grow in

association with hetero-fermentative lactic acid bacteria, enhancing the accumulation of specific aroma compounds, including alcohols, lactones, and medium-chain fatty acids (MCFAs) (Gänzle, Ehmann, & Hammes, 1998; Gobbetti, 1998). Results show that the combination of a protein-reticulating enzyme and sourdough on wheat bread produced a positive synergistic effect.

4.2. Materials and Methods

4.2.1. Raw Materials and Chemicals

Straight-grade wheat flour was provided by Barilla S.p.A. (Parma, Italy). The transglutaminases tested in this study came from different sources: (i) Activa® WM (acTG), from *Streptoverticillum mobaraense*, was purchased from Ajinomoto (Mesnil-Saint-Nicaise, France), (ii) a recombinant microbial transglutaminase (zTG) was purchased from Zedira (Darmstadt, Germany), (iii) a mammalian transglutaminase, from guinea pig liver, was purchased from Sigma-Aldrich (Milan, Italy), and (iv) a recombinant transglutaminase of plant origin, the *Arabidopsis thaliana* peptide N-glycanase (AtPng1p), was purified as previously described (Della Mea, Caparros-Ruiz, & Rigau, 2004).

All reagents and solvents (unless otherwise indicated) were of the highest purity and were obtained from Sigma-Aldrich (Milan, Italy).

4.2.2. Sourdough Preparation

Sourdough was prepared by a two-step fermentation process using *L. sanfranciscensis* strain, LSCE1 and *C. milleri* strain, PFL44 both belonging to the Department of Agricultural and Food Science, University of Bologna (Italy). Strains were grown in mMRS medium (Stolz, Bocker, Hammes, & Vogel, 1995) and

Sabouraud Dextrose medium (Oxoid, Basingstoke, UK), respectively. The dough was prepared by mixing wheat flour and water to reach a dough yield (DY) of 220. Fermentation was performed in a fermentor (BioFlo/CelliGen 115, New Brunswick, Eppendorf) as previously described (Scarnato, Serrazanetti, Aloisi, Montanari, Lanciotti, & Del Duca, 2016). The sourdough obtained was used for the preparation of bread in association or not with transglutaminase.

4.2.3. Enzymatic Treatment with TGs

In order to induce protein cross-links, different amounts of transglutaminase (0.5, 1, 2, and 5 U/g flour) were added to the wheat dough obtained using *Saccharomyces cerevisiae* as a conventional leavening agent and the sourdough made with the selected microbial consortium described above. The enzyme was mixed to the flour for 15, 60 or 90 min at different temperatures, from 4 to 37 °C.

Cross-linking was evaluated by protein extraction and separation using 15% SDS-PAGE. Total proteins were extracted under reducing conditions using the buffer described by (Marco, Perez, Ribotta, & Rosselli, 2007). Albumins/globulins (F1), prolamins (F2), and glutelins (F3) were extracted following a sequential extraction method using different solvents (Marco, Perez, Ribotta, & Rosselli, 2007). Globulins 7S and 11S were prepared and purified as previously described (Thanh, Okubo, & Shibasaki, 1975). The protein content of the extracts was determined by the bicinchoninic acid method (Smith, Krohn, Hermanson, Mallia, Gartner, Provenzano, et al., 1985).

4.2.4. Measurement of TG specific activity

TG specific activity was measured by the conjugation of BC to protein substrates as previously described (Lilley, Skill, Griffin, & Bonner, 1998) with slight

modifications. Protein substrates, such as standard proteins and wheat protein extracts, were covalently attached to the surface of microplate wells. The level of BC incorporation was determined according to an established protocol (Lilley, Skill, Griffin, & Bonner, 1998) with 10 mM cystamine replacing EDTA in negative controls. The enzyme specific activity was expressed as a 0.1 change in A450 per h per mg of enzyme used (U/mg prot.).

4.2.5. Dot-blot of Enzyme Reaction Products

Fractions F1, F2, and F3 were treated with transglutaminase and the reaction products were blotted onto nitrocellulose. The membrane was incubated with Ab3, a monoclonal antibody raised against soluble transglutaminase (Neomarker, Fremont, CA, U.S.A.) and with 81D4, a monoclonal anti-Nε (γ-glutamyl)-lysine antibody (Covalab, Lyon, France), which is a product of the transglutaminase cross-linking reaction. Dots were revealed using horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and 3-amino-9-ethylcarbazole (AEC).

4.2.6. Bread Preparation

Bread was prepared with a bread maker (Deluxe Princess, 152000) using an industrial recipe (wheat flour, water, sugar, salt, baker's yeast, and extra-virgin olive oil) to obtain a final volume of about 500 g with DY 150. When sourdough was used, it was added at a concentration of about 30% of the final weight of dough; the amount of flour and water was reduced accordingly in order to maintain the same DY. Transglutaminase was added at different concentrations (0.5, 1, and 2 U/g flour). Doughs were kneaded for 14 min and fermented for 20 min. Then, a second kneading of 8 min was performed, followed by 1 h of fermentation. Dough samples,

and sourdough with and without transglutaminase were then baked at 180 °C for 30 min in order to obtain conventional bread and sourdough bread.

4.2.7. Bread Empirical Rheological Properties

Bread mechanical characteristics were evaluated with a Texture Analyzer mod. TA.HDi 500 (Stable Micro System, Godalming, Surrey, UK) equipped with a P/20 mm aluminium cylinder probe and a 25 kg load cell. For each sample, three slices of 15 mm were cut from the central portion of two different bread loaves. Texture profile analysis (TPA) and stress relaxation tests were performed. The TPA test in a double compression cycle was performed as described earlier (Gàmbaro, Varela, Gimenez, Aldrovandi, Fiszman, & Hough, 2002). Four textural parameters, expressed as hardness, resilience, cohesiveness, and chewiness of the crumb were used as indicators of structural characteristics. The stress relaxation test was performed as described by (Stollman & Lundgren, 1987); texture parameters, such as hardness and springiness, were determined.

4.2.8. Bread Image Analysis

The inner portion of bread slices was used for crumb grain features measurements. A digital camera mod. D7000 (Nikon, Shinjuku, Japan) was used to acquire digitalized images of samples placed inside a black box under controlled lighting conditions. The slice images were spatially calibrated using Image Pro-Plus v. 6.2 (Media Cybernetics, USA). Grey levels of scanned slices were evaluated by the model in terms of percentage holes and crumb area over the total. The percentage crumb porosity on the total alveolation of the slice portion was determined. Moreover, crumb morphological features were evaluated including the cell area distribution. Holes were identified, counted and classified into four predefined area

classes on the basis of their size (cm2): 0.00025 < class 1 < 0.025; 0.025 < class 2 < 0.25; 0.25 < class 3 < 0.5; 0.5 < class 4 < 1. Finally, the aspect ratio was calculated by dividing the width by the height of the bread slices (Collar & Angioloni, 2014).

4.2.9. Bread Shelf-Life Evaluation

After a 2-h cooling period, bread samples (conventional and sourdough breads) were introduced in polyethylene plastic bags in ordinary atmosphere, sealed and stored at room temperature (24±2 °C) for 15 days without preservatives. Samples were monitored daily to check for the presence of spoilage microflora (molds and *Bacillus spp.*); microbial growth was tested by plate counting after 7 and 15 days. Analyses were performed on Malt Extract Agar (Oxoid, Basingstoke, UK) for molds and on Plate Count Agar (Oxoid, Basingstoke, UK) for *Bacillus spp.*

4.2.10. SPME-GC-MS Analysis

Volatile compounds (VCs) were monitored by using Gas Chromatography-Mass Spectrometry coupled with solid phase micro-extraction (SPME-GC-MS). Doughs (conventional and sourdough) and baked samples (conventional and sourdough) were placed in sterilized vials and 4-methyl-2-pentanol (final concentration 33 mg/kg) were added as the internal standard. Samples were pre-equilibrated for 10 min at 47 °C and then a fused silica fibre (DVB/CAR/PDMS StableFlex, Supelco, Steiheim, Germany), was introduced in the head-space for 40 min. Molecules were detected using the method described by (Montanari, Bargossi, Lanciotti, Chinnici, Gardini, & Tabanelli, 2014). Identification was based on the comparison of mass spectra with those of NIST and Wiley databases. Volatile compounds were expressed as a percentage of the total peak area. Identification of MCFAs was also confirmed by comparing their retention times with those of pure

compound mixtures (e.g., BAME Mix, Sigma Aldrich, Italy). Only identified compounds reaching concentrations above 0.2% of the total peak area were used for subsequent Principal Component Analysis (PCA) according to (Patrignani, Montanari, Serrazanetti, Braschi, Vernocchi, Tabanelli, et al., 2016). However, a preliminary one-way analysis of variance (ANOVA; significance P ≤0.05) was performed to confirm that the excluded peaks were not significant for sample characterization.

4.2.11. Statistical Analysis

The data are the means of three replicates and are expressed as mean ± SD. The data were examined using one- and two-way ANOVA with a significant difference of at least 95% according to the Bonferroni post-test (GraphPad Prism software), **= $p \leq 0.01$.

Textural data were compared by the Kruskal-Wallis test in case of significance with the Levene test ($p <0.05$) (Statistica 8.0, StatSoft Inc., Tulsa, OK, U.S.A).

4.3. Results

4.3.1. Comparison of TG Activities on Different Protein Substrates

Initially, the four different transglutaminases (acTG; zTG; gplTG and AtPng1p) were tested on well-known protein substrates of transglutaminase, such as dimethylcasein (DMC), fibronectin (FN), and some protein fractions present in flours, i.e., 7S and 11S globulins (from soybean), prolamins, gluten, and glutenins from wheat. All the transglutaminases showed activity on the tested substrates. The two transglutaminases of microbial origin (acTG and zTG) showed the highest specific activity that reached 4000 U/mg prot

(Figure 4.1 A). gplTG also showed high activity on these substrates with activities > 1000 U/mg prot. Moreover, the two microbial transglutaminases showed the highest activity on gluten and its components, gliadins and glutenins. When 11S globulins, glutenins, and gliadins were incubated with the various enzymes at different temperatures for 90 min in order to evaluate their cross-linking effect, varying degrees of substrate aggregation were visible by SDS-PAGE in regions corresponding to high molecular weight (HMW), compared to the same protein not treated with the enzyme (Figure 4.1 B).
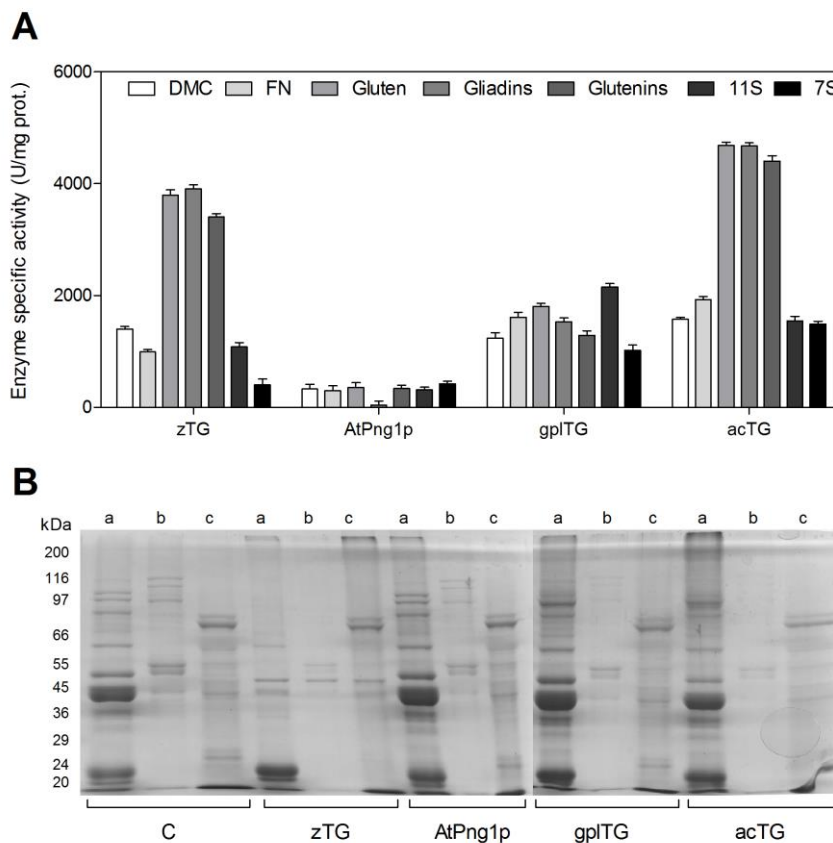
Figure 4.1. TG activity on several protein substrates and consequent formation of HMW products; A) specific enzyme activity, assayed by the microplate biotin cadaverine incorporation method, of mammalian (gplTG), microbial (acTG and zTG), and plant (AtPng1p) TGs; DMC, N', N'-dimethyl casein; FN, fibronectin; 7S, vicilin from soy; 11S, legumin from soy. B) Coomassie stained SDS-PAGE showing protein cross-linking effects of TGs on different storage proteins at 30 °C for 90 min of reaction; a= 11S, b= gluten, c= prolamins.

At 30 °C, AtPng1p, gplTG, and acTG were efficient in the cross-linkage of 11S globulin. Both microbial transglutaminases were very effective in reticulating all the tested substrates in a wide range of temperatures (4-37 °C). As acTG showed the highest activity on the different protein substrates, with major aggregating effects at 30 °C, it was chosen for subsequent experiments.

4.3.2. acTG-mediated Cross-Linking in Dough

As a first attempt to investigate the cross-linking activity of acTG on wheat dough, different enzyme dosages (0.5, 1, 2, and 5 U/g flour) were added. The formation of HMW proteins was dependent on enzyme dosage, mixture time, and water amount. In order to acquire information about the proteins involved in the cross-links, different protein fractions (F1, F2, and F3) were extracted from dough. Protein separation by SDS-PAGE showed that acTG, increased the formation of HMW products mostly in F2 and F3 (Figure 4.2 A, brackets).
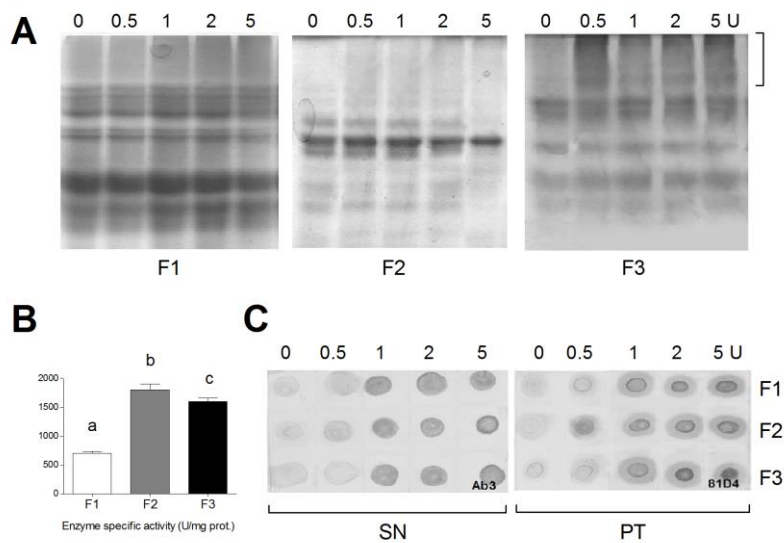
Figure 4.2. Dose-dependent cross-linking effects of acTG on wheat flour protein fractions. A) Electrophoretic patterns of wheat dough protein fractions enriched in albumins/globulins (F1), prolamins (F2), and glutelins (F3), treated with different amounts of acTG for 90 min; B) specific enzyme activity of acTG on F1, F2, and F3. C) Dot blot analysis of F1, F2, and F3 treated with different amounts of acTG and probed with the Ab3 antibody against TG (supernatant, SN) and 81D4 against cross-links (pellet, PT).

This was confirmed by the enzyme activity assay, showing that F2 and F3 were the best acTG substrates as measured by incorporation of cadaverine (Figure 4.2 B). Moreover, the immune-recognition of the signal corresponding to acTG increased in supernatants while the appearance of cross-links increased in the pellets, as shown in dot blot experiments. F2 showed cross-linked products at the lowest acTG concentration (0.5 U/g flour) (Figure 4.2 C).

4.3.3. Combined Effects of acTG and Sourdough on Proteins

The formation of HMW products, both in dough and sourdough samples, was analysed by separation of the total protein extract before and after acTG treatment. As shown in Figure 4.3 A (bracket), HMW products were enhanced by the presence of the sourdough.
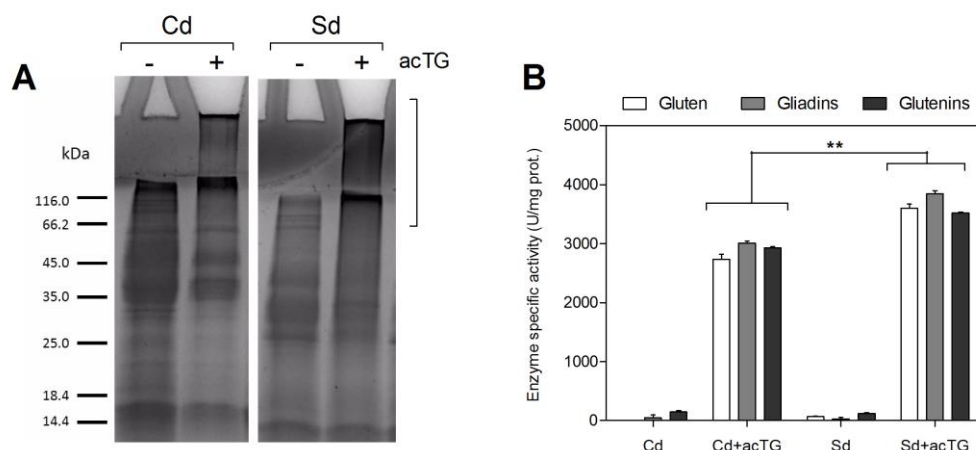

Figure 4.3 Combined effects of acTG and sourdough on wheat dough proteins. A) SDS-PAGE highlighting HMW products (brackets) in total protein extracts from doughs treated with 0.5 U of acTG and sourdough; Cd=conventional dough, Cd+acTG=conventional dough plus acTG, Sd=selected sourdough, Sd+acTG=selected sourdough plus acTG; B) biotin cadaverine incorporation

assay on wheat protein fractions (F1, F2 and F3) in the presence of acTG and sourdough; Cd=conventional dough, Cd+acTG= conventional dough plus acTG, Sd=selected sourdough, Sd+acTG= selected sourdough plus acTG.

TG activity on fractions F1, F2, and F3 extracted from dough and sourdough confirmed that the presence of sourdough significantly increased enzyme activity in a synergistic way as compared with sourdough alone, which was not active (Figure 4.3 B).

4.3.4. Physicochemical and Rheological Features of Baked Samples

As reported in Figure 4.4 and Table 4.1, the conventional bread was the softest sample, as it exhibited the lowest hardness value (248 g) as compared with the other samples. With increasing amounts of acTG, the baked samples reached hardness values of 642 g, 1060 g, and 1177 g with 0.5 U, 1 U, and 2 U of enzyme, respectively. Hardness was significantly reduced to values around 500 g when the same units of enzyme were added to sourdough bread.

Figure 4.4. Crumb morphological features of baked bread samples analysed by TPA and Relaxation test. Effect of acTG and sourdough on baked samples rheological properties; Cb=conventional bread, Cb+acTG=conventional bread supplied with acTG, Sb=sourdough bread, Sb+TG=sourdough bread supplied with acTG. Values are means ± SD of three replicates experiments. Means (n = 3) followed by the same letter within the same column are not statistically different by using the Kruskal-Wallis in case of significance of the Levene test (p <0.05).

A similar trend in the chewiness parameter was observed as the forces values rose after the addition of acTG, while in sourdough bread this value

remained almost constant even after enzyme addition. Samples made with sourdough and acTG had lower hardness and chewiness values compared to conventional bread plus enzyme in a dose-dependent manner. Conventional bread showed the highest value of cohesiveness and resilience, freshness index, and softness. A reduction of these values was obtained by increasing the enzyme units, while adding the sourdough had no significant effects because it counteracted those induced by the enzyme.

Results obtained in the stress relaxation test showed a similar trend to those obtained in the TPA, confirming the synergic benefit of sourdough in combination with acTG (Table 4.1).

Bread samples	TPA test				Relaxation test	
	Hardness (g)	Cohesiveness	Resilience	Chewiness (g)	Hardness (g)	Springiness (%)
Cb	248± 27 b	0.80±0.02 a	0.47±0.01 a	197±18 b	193±27 b	55±0.58 a
Cb+ acTG (0.5 U)	642±39 ab	0.69±0.02 ab	0.38±0.03 ab	439±18 ab	367±28 ab	48±3.08 a
Cb+ acTG (1 U)	1060±95 a	0.67±0.01 ab	0.36±0.01 ab	709±74 a	655±42 ab	49±1.21 a
Cb+ acTG (2 U)	1177±166 a	0.62±0.05 ab	0.28±0.04b	724±61 a	715±52 a	45±5.33 a
Sb	456±5 ab	0.67±0.01 ab	0.36±0.02 ab	307±9 ab	334±23 ab	43±1.69 a
Sb+ acTG (0.5 U)	548±96 ab	0.60±0.04 b	0.31±0.05 ab	331±70 ab	333±44 ab	45±2.61 a
Sb+ acTG (1 U)	368±51 ab	0.71±0.02 ab	0.46±0.02 a	260±32 ab	213±32 ab	45±2.56 a
Sb+ acTG (2 U)	494±43 ab	0.67±0.03 ab	0.40±0.02 ab	330±14 ab	210±21 ab	45±1.44 a

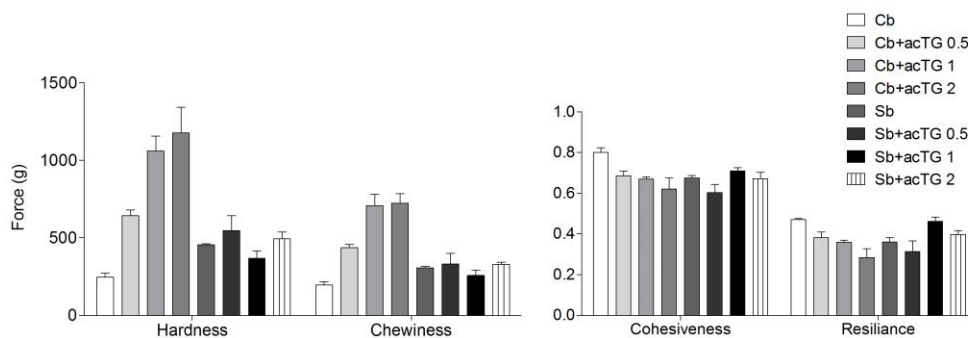Table 4.1 Crumb morphological features of baked bread samples analysed by TPA and Relaxation test. Effect of acTG and sourdough on baked samples rheological properties; Cb=convention al bread, Cb+acTG=convention al bread supplied with acTG, Sb=sourdough bread, Sb+TG=sourdough bread supplied with acTG. Values are means ± SDof three replicates experiments. Means (n = 3) followed by the same letter within the same column are not statistically different by using the Kruskal-Wallis in case of significance of the Levene test (p <0.05).

4.3.5. Crumb Morphological Features of Baked Samples

Bread crumb gas cell size of baked samples were investigated as they have a significant effect on bread texture and on mouth feel perception. As reported in Table 4.2, similar porosity values, expressed as percentage of hole areas, were observed in all bread slices. All samples were characterized by a fine crumb, i.e., gas cells belonging to classes 1 and 2 in most of the area. The

effect of sourdough and acTG (all concentrations) combined caused an increase in class 2 gas cells but not class 1.

The aspect ratio, an index of product volume, showed that acTG caused a volume reduction of the slice in a dose-dependent manner. The presence of sourdough, in combination with acTG, increased the volume of the slice, bringing aspect ratio values close to the control sample (Figure 4.4, and Figure 4.5).
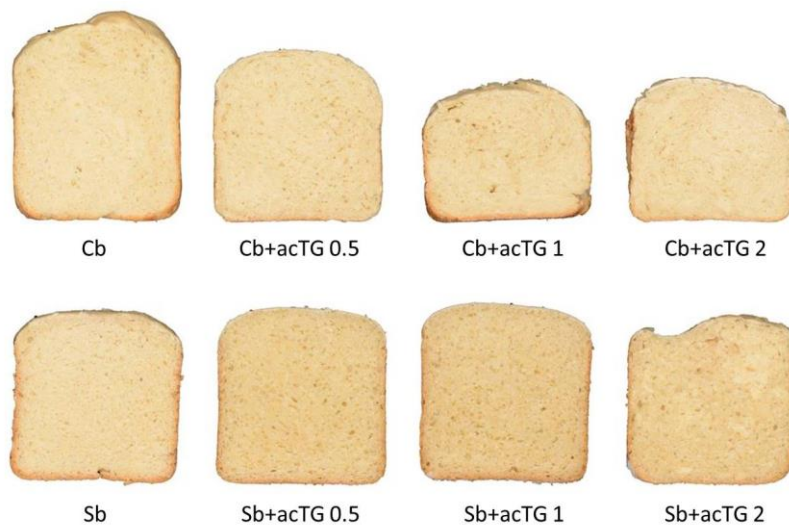
Figure 4.5 Representative images of baked sample slices obtained with and without sourdough and different amounts of TG (0.5, 1 and 2 U/g flour); Cb=conventional bread, Cb+acTG=conventional bread supplied with acTG, Sb=sourdough bread, Sb+TG=sourdough bread supplied with acTG.

In particular, conventional bread had the lowest aspect ratio value (0.83±0.04), which increased in a dose-dependent manner in samples treated with increasing acTG units, reaching values of 1.26±0.06. sourdough breadhad an aspect ratio of 1.00±0.03, while the addition of 1 U of acTG slightly decreased this value to 0.96±0.01 (Table 2).

Bread samples	Area holes (%)	Area crumb (%)	Classes	Holes (%)	Area (%)	Aspect ratio
Cb	17.55±3.57	82.45±3.57	1	97.81±0.74	50.36±14.45	0.83±0.04
			2	2.02±0.60	30.98±2.85	
			3	0.11±0.10	7.90±5.61	
			4	0.06±0.04	10.76±5.98	
Cb+acTG 0.5	11.28±1.28	88.72±1.28	1	97.57±0.52	65.81±2.65	0.96±0.03
			2	2.43±0.52	34.19±2.65	
			3	0.00	0.00	
			4	0.00	0.00	
Cb+acTG 1	12.39±3.07	81.74±0.50	1	98.31±0.07	55.73±1.97	1.26±0.06
			2	1.61±0.08	28.81±1.17	
			3	0.04±0.06	6.62±9.37	
			4	0.03±0.05	8.84±12.50	
Cb+acTG 2	18.26±0.50	81.74±0.50	1	97.77±0.59	52.94±4.90	1.17±0.02
			2	2.11±0.55	35.24±2.10	
			3	0.06±0.08	4.35±6.15	
			4	0.04±0	7.47±3.35	
Sb	17.18±1.08	82.82±1.08	1	97.08±0.68	57.40±11.72	1.00±0.03
			2	2.92±0.68	42.60±11.72	
			3	0.00	0.00	
			4	0.00	0.00	
Sb+acTG 0.5	26.79±1.06	73.21±1.06	1	94.66±0.10	24.35±4.78	1.02±0.01
			2	4.70±0.09	40.04±7.31	
			3	0.41±0.01	18.98±4.69	
			4	0.21±0.21	16.63±16.78	
Sb+acTG 1	18.27±1.16	81.73±1.16	1	93.57±0.55	37.33±4.83	0.96±0.00
			2	6.37±0.47	59.37±0.15	
			3	0.06	3.31±4.68	
			4	0.00	0.00	

Table 4.2 **Bread crumb gas cell size and aspect ratio.** Effect of acTG and sourdough on crumb morphological features and aspect ratio of baked samples. Holes were identified, counted and classified into 4 predefined area classes on the basis of their size (cm2): 0.00025 < class 1 < 0.025; 0.025 < class 2 < 0.25; 0.25 < class 3 < 0.5; 0.5 < class 4 < 1; Cb=conventional bread, Cb+acTG=conventional bread supplied with acTG, Sb=sourdough bread, Sb+TG=sourdough bread supplied with acTG. Values are means ± standard SD of three replicates experiments.

4.3.6. Combined Effect of acTG and Sourdough on VCs

The volatile compound profiles of dough and breads, both conventional bread and sourdough breadtreated or not with acTG, were determined by GC-MS-SPME. This approach has proven its potential in providing a volatile compound fingerprint of food and beverages in relation to their microbiota, composition, and/or production processes (Montanari, Bargossi, Gardini, Lanciotti, Magnani, Gardini, et al., 2016; Patrignani, et al., 2016; Scarnato, Serrazanetti, Aloisi, Montanari, Lanciotti, & Del Duca, 2016). Sixty molecules belonging to different chemical classes were identified and specific volatile compound fingerprints were obtained in relation to the treatment and before and after baking. In particular, conventional bread was mainly characterized by ethanol, phenylethyl alcohol, and furanones. The addition of transglutaminase and sourdough caused a significant modification of the profile. Since profiles were very complex, data was subjected to PCA. All the samples were mapped in the space spanned by the first two principal components, with PC1 and PC2 explaining 35.48 and 26.00% of the variance, respectively. The score plot showed that samples were clustered mainly according to the baking process and the presence of sourdough (Figure 4.6 A). Samples were separated into four clusters; cluster 1 grouped conventional bread samples with and without acTG, while cluster 2 comprised sourdough breadsamples, independent of the enzyme addition. Clusters 3 and 4 included sourdough and conventional dough, respectively.
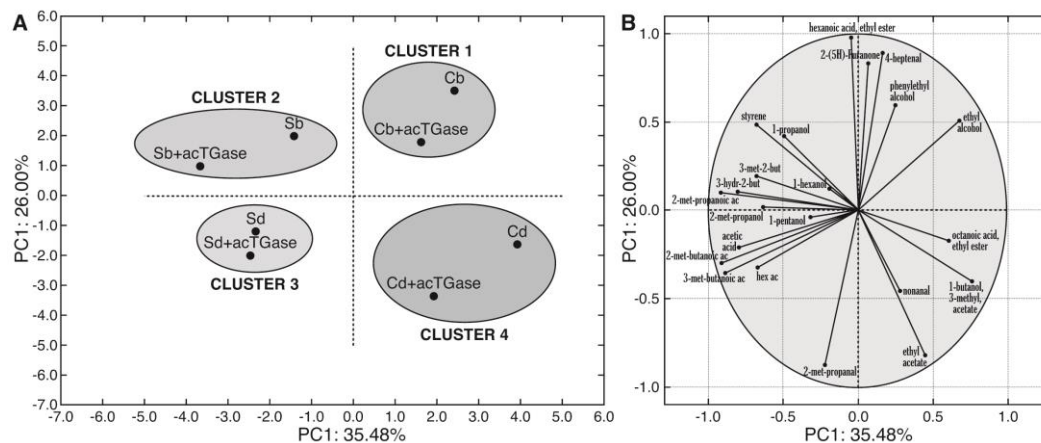
Figure 4.6. Principal Component Analysis of volatile compounds detected in the different doughs (before baking) or breads obtained with or without acTG (0.5 U/g flour) and with or without sourdough: Cd=conventional dough, Cd+TG= conventional dough supplied with acTG, Cb=conventional bread, Cb+acTG=conventional bread supplied with acTG, Sd=selected sourdough, Sd+acTG=selected sourdough supplied with acTG, Sb=sourdough bread, Sb+TG=sourdough bread supplied with acTG. In particular: A) PCA score plot on the two first factors (PC1 and PC2); B) loadings plots of the VCs obtained from the PCA. Abbreviations: 2-methyl-butanoic acid: 2-met-butanoic ac; 3-methyl-butanoic acid: 3-met-butanoic ac; hexanoic acid: hex ac; 3-methyl-2-butanone: 3-met-2-but; 3-hydroxy-2-butanone: 3-hydr-2-but; 2-methyl-propanoic acid: 2-met-propanoic ac; 2-methyl-propanal: 2-met-propanal

The fermentation process separated samples as sourdough and sourdough bread along PC1 regardless of the presence of the enzyme. The baking process differentiated the samples, conventional and sourdough breads along PC2. As shown by the loading plots (Figure 4.6 B), the presence of sourdough strongly affected volatile compound profiles independent of the baking process and enzyme addition. In fact, clusters 2 and 3 were characterized by the highest contents of molecules deriving from the activities of sourdough microbiota, including ketones, MCFAs, and alcohols. Volatile compounds such as furanone and phenyl ethyl alcohol characterized the baked samples.

4.3.7. Shelf-Life of Baked Products

In order to evaluate the impact of sourdough fermentation on shelf-life, the baked samples were monitored during 15 days of storage at room

temperature. A relevant extension (up to 15 days) of the shelf-life, in terms of microbial growth prevention (namely of molds and *Bacillus* spp), of bread obtained with sourdough and acTG addition was observed. After 3 days of storage, the samples did not appear visibly different. After 7 days, conventional bread was characterized by the presence of molds on the surface. The microbiological analyses, performed at this sampling point, revealed levels of molds and *Bacillus spp.* higher than 5 log CFU/g, that reached about 8 log CFU/g after 15 days of storage. Differently in sourdough breadplus acTG these species remained under the detection limit after 7 days, while 3 log CFU/g were detected after 15 days of storage (Figure 4.7 A, and B).
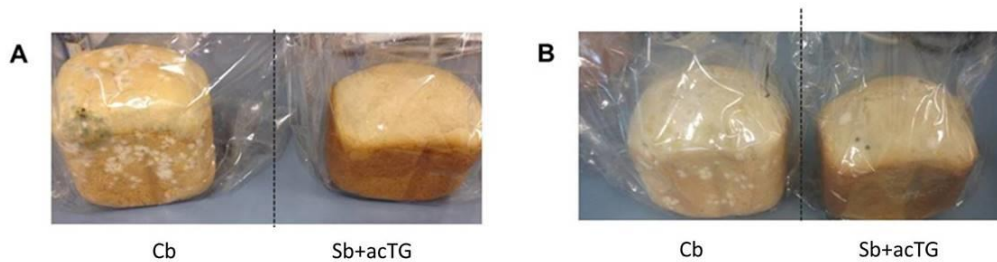
Figure 4.7 Shelf-life of baked products obtained using sourdough and 0.5 U/g flour of acTG (Sb+acTG), compared to conventional bread (Cb); A) breads after 1 week of storage at room temperature (24±2 °C); B) breads after 2 weeks of storage at room temperature.

4.4. Discussion

In this work, both the acTG enzyme and the microbial consortium consisting of *L. sanfranciscensis* and *C. milleri* alone showed advantages and disadvantages, the latter compensated by the supplementation of the two agents together. The final product showed enrichment in flavor and improved rheological properties.

The interaction of microbially-produced metabolites or flavor compounds with bread microstructure is generally underestimated and, therefore, not systematically investigated. However, (Aponte, Boscaino, Sorrentino, Coppola, Masi, & Romano, 2014), studying the effect of fermentation time on the microstructure and volatile compounds of chestnut flour-based sourdough, showed a significant relationship between the protein network surrounding starch globules and the volatile compound composition of sourdough. Consequently, the specific volatile compound fingerprints obtained here could also be the result of changes induced in the protein network by transglutaminase and/or enzymatic activities of sourdough microflora.

The addition of acTG led to detrimental effects on conventional bread. The high hardness and chewiness values of conventional bread treated with acTG was, however, counteracted by using sourdough obtained with the selected microbial consortium. These findings are in agreement with the hydrolysis of protein networks that occurs during sourdough fermentation, probably due to a pH-mediated activation of cereal proteolytic enzymes (Loponen, Mikola, Katina, Sontag-Strohm, & Salovaara, 2004). It is known that lactic acid bacteria may affect product rheology through a strain-dependent proteolytic activity (Gobbetti, Smacchi, & Corsetti, 1996). On the other hand, degradation of wheat gluten would reduce the viscoelastic properties, responsible for the leavening capacity of bread and other baked products.

The main benefits deriving from the use of the microbial consortium were enriched in flavors, leavening and increased in shelf-life. Moreover, it is

well known that sourdough contributes to the quality of baked goods as it is rich in nutrients and other compounds with beneficial health effects (De Vuyst, Van Kerrebroeck, Harth, Huys, Daniel, & Weckx, 2014). Under our experimental conditions, sourdough increased the accumulation of some molecules, such as ketones, MCFAs, and alcohols. These findings are in agreement with evidences underlining that 1-propanol, 3-methyl-2-butanone, 3-hydroxy-2-butanone, and 2- and 3-methyl-butanoic acid are the main volatile compounds produced by selected lactic acid bacteria isolated from sourdough (Damiani, Gobbetti, Cossignani, Corsetti, Simonetti, & Rossi, 1996). Moreover, the interaction between yeast and lactic acid bacteria in sourdough results in a significant enhancement of bread aroma profiles (Gänzle, Vermeulen, & Vogel, 2007; Hansen & Schieberle, 2005; Kirchhoff & Schieberle, 2001). The proteolytic system of lactic acid bacteria releases low-molecular-weight peptides and amino acids, which promote the development of metabolic activity in the microorganisms, helping to obtain an improved taste and flavor due to their further production of aroma compounds (Di Cagno, De Angelis, Alfonsi, De Vincenzi, Silano, Vincentini, et al., 2005; Rizzello, De Angelis, Di Cagno, Camarca, Silano, Losito, et al., 2007). The aroma profile of both dough and baked bread also results from osmotic stress. In fact the exposure of microbial cells to stressful conditions during the fermentation process induces a broad transcriptional response (Serrazanetti, Guerzoni, Corsetti, & Vogel, 2009), reported to affect the organoleptic properties via several metabolic activities (Guerzoni, Vernocchi, Ndagijimana, Gianotti, & Lanciotti, 2007). In particular, it has been reported that *L. sanfranciscensis* in sourdough responds to environmental stresses, with

an overproduction of specific compounds such as 3-methyl butanoic acid and 2(5H)-furanones (Erasmus, Van Der Merwe, & Van Vuuren, 2003; Guerzoni, Vernocchi, Ndagijimana, Gianotti, & Lanciotti, 2007).

Concerning the effect of transglutaminase on bread shelf-life, Gottardi et al. (2014) reported that some peptides from gluten obtained through the activity of transglutaminase showed antimicrobial activity (Gottardi, Khoon Hong, Ndagijimana, & Betti, 2014). The release of such peptides can play a role in the modulation of bread microbiota during the storage and consequently on final product shelf-life. Caballero et al. (2007) showed an increase of staling when this enzyme is used in gluten-based bread. However, Collar and Bollain (2005) reported that the combined use of transglutaminase and α-amylase reduced crumb staling kinetics and sensory deterioration (Collar & Bollan, 2005). In our experimental conditions, the use of sourdough assured the presence of α-amylase released by microbial activity (Sieuwerts, de Bok, Hugenholtz, & van Hylckama Vlieg, 2008), and therefore mitigated the bread staling properties of transglutaminase.

4.5. Conclusions

In this work, the synergistic beneficial effects on bread characteristics of microbial acTG and a consortium of *L. sanfranciscensis* and *C. milleri* in bread-making are described. The excessive hardness and chewiness of conventional bread caused by increasing concentrations of acTG were counteracted by the addition of a proper amount of sourdough. On the other hand, the degradative action of sourdough on protein substrates, which reduces the viscoelastic properties of bread, was compensated by the protein-aggregating

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effect of transglutaminase. In summary, the use of sourdough combined with acTG showed positive effects on bread rheological features, shelf-life, and aroma profile.

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5. Immunoreactivity of Flour Proteins After Enzymatic Treatment

This chapter is based on:

Scarnato, L.; Gadermaier, G.; Ferreira, F.; Caio, G.; De Giorgio, R.; Del Duca, S. *Study of Allergenic Compounds in Gluten and Gluten-free Flours Treated with Biotechnological Processes for Bread Making* (2017)

Abstract

The aim of this work was to evaluate the effect of addition of microbial transglutaminase, on protein aggregation in flours as well as in baked products. In this study, the effect of microbial transglutaminase treatment on different flours from wheat and gluten-free sources (e. g. rice, corn, and amaranth), was analysed in order to evaluate the capacity to modify protein substrates. For this purpose, doughs were treated by adding 1U of enzyme per 100 mg flour. The reaction was carried out for 90 min at 40 °C. To investigate the enzyme effect on the formation of protein cross-linked products, three sequential protein fractions, corresponding to

albumins/globulins, prolamins and glutelins, were extracted and analysed by SDS-PAGE. Results showed that microbial transglutaminase has the capacity to modify gluten-free flour proteins and to increase the amount of protein aggregates clearly visible on the SDS-PAGE as high-molecular weight proteins. Determination of gluten content in wheat samples was carried out by R5-based assays that detect gluten specific amino acid sequences. In order to evaluate structural epitopes of the flour proteins upon microbial transglutaminase treatment, enzyme-linked immunosorbent assay (ELISA) was performed. Therefore, sera from celiac disease, non-celiac gluten sensitive patients and healthy donors were analysed with respect to their IgG-binding capacities. Detection of IgG-binding epitopes was conducted with an alkaline phosphatase labeled anti-human IgG antibody and signals before and after enzyme treatment were compared. Data showed that no significant difference was observed in the immunoreactivity of the wheat protein extracts after enzyme treatment. These preliminary results give a perspective in the gluten-free research and suggest their possible use to create innovative products.

5.1. Introduction

Celiac disease and other non-celiac gluten sensitivities (NCGS) are immune-mediated systemic disorders, elicited by gluten and related prolamines. Recent epidemiological studies have shown that 1% of the European population suffer from celiac disease (Kurien, Trott, & Sanders, 2016). Such a ratio establishes that gluten related disorders are one of the most common food intolerances. The gliadin protein fraction of gluten is the main factor responsible for the development of the disease. Sensitive patients eating wheat or related proteins, such as hordeins (barley) or secalins (rye) undergo an immunological response, localized in the small intestine,

which destroys mature absorptive epithelial cells on the surface of the small intestine. Currently, the only therapeutic option for people suffering from celiac disease and other gluten sensitive disorders is lifelong adherence to a strict gluten-free diet (Dowd, Tamminen, Jung, Case, McEwad, & Beauchamp, 2014). European Regulation concerning the composition and labelling of foods suitable for people intollerant to gluten, indicates that the gluten-free foodstuffs must contain less than 20 mg/kg of gluten in the finished product[2]. In agreement with the Codex Alimentarius Commission, a highly sensitive and specific technique for gluten analysis in food is based on the R5 antibody. This is an immunological test based on the R5 monoclonal antibody recognizing potential celiac toxic epitope present in wheat gliadins. The R5 epitope LQPFP is present in the 33-mer sequence, LQ<u>LQPFP</u>QPQLPYPQPQLPYPQPQLPYPQPQPF, a highly protease-resistant region present in α-gliadins in wheat that is shown to be highly immunogenic in celiac disease (Shan et al., 2002).

However, gluten is also the major factor involved in the structural properties of bakery products. Due to its ability to create aggregates, it gives dough viscosity, elasticity and cohesion, all features responsible for the baking performance (Delcour, Joye, Pareyt, Wilderjans, Btijs, & Lagrain, 2012). Due to the unique properties of gluten, it is a huge challenge for food scientists to produce high quality gluten-free products.

Cross-linking enzymes, able to organize and create protein networks, are suitable protein modifiers for the food industry. Among those enzymes,

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[2] Commission Regulation (EC) N. 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten.

transglutaminase can be used for protein manipulation in order to achieve higher food quality due to its ability to improve the firmness, viscosity, elasticity and water-binding capacity of food products (Kieliszek & Misiewicz, 2014). These positive effects are associated with the formation of inter or intra-molecular cross-links that can modify structural characteristics of poor flour dough. For these reason it is interesting to evaluate the protein composition and nutritional safety of the gluten-free flours before and after the above-mentioned biotechnological process. Considering that transglutaminase is not only able to lead protein changes by cross-linking reaction affecting the protein structural net and composition, but also to catalyse the deamidation of glutamine residue, the effect of enzyme treatment may in theory lead to "immunotoxic" food. The complexity of this biological process could have effects on the health of the consumers. Changes of the raw materials could modify their antigenic properties and capacity to cause immunogenic reactions in susceptible individuals (Ruh, Ohsam, Pasternack, Yokoyama, Kumazawa, & Hils, 2014). Changes in the food allergenicity potential after processing procedures was identified early in the history of food allergy research, through the report of Prausnitz and Kustner (1921) on the fish-based food sensitivity after cooking (Prausnitz & Kustner, 1921). The processing procedures can alter the structure and properties of food proteins in poorly defined ways, which may in turn affect the ability of a given protein to act as an allergen by either sensitizing in individuals or eliciting an allergic reaction.

For these reasons, it would be important to evaluate the molecular effects on the protein pattern during food processing and assess health hazards.

The aim of this research was to evaluate the capacity of microbial transglutaminase, from *Streptoverticillium mobaraense* to modify protein substrates of wheat and gluten-free flours by catalysing protein cross-links. The enzyme activity on protein substrates determine rearrangement of protein structures which govern new textural attributes. Such structures may affect the immunogenic potential of the proteins. In order to evaluate the potential epitope changes, the IgG immunoreactivity was investigated in the dough after processing.

5.2. Materials and Methods

5.2.1. Flours and Sourdough

Gluten and gluten-free flours were from several sources belonging to cereals (wheat, rice, and corn), pseudo-cereals (amaranth). The microbial transglutaminase tested in this study, Activa WM (acTG) from *Streptoverticillum mobaraense*, was a kind gift of Ajinomoto (specific activity: 81–135 U/g, Ajinomoto Foods Europe S.A.S., France). Sourdough was prepared, as reported before with a two-step fermentation using *Lactobacillus sanfrancisciensis* (LSCE1) and *Candida milleri* (PFL44), for 21 hrs of fermentation (Scarnato, Serrazanetti, Aloisi, Montanari, Lanciotti, & Del Duca, 2016).

All reagents and solvents were analytical grade and purchased from Sigma-Aldrich (Milan, Italy), unless otherwise stated.

5.2.2. Sera

A collection of human sera from celiac disease (CD) (n=14), non-celiac gluten sensitive (NCGS) patients (n=17), and healthy control blood donors (HCBD) (n=6), were kindly provided by the Departments of Medical and Surgical Sciences and Digestive System of the Bologna University, St. Orsola-Malpighi Hospital, Bologna, Italy. Patients with gluten sensitivity were selected on the basis of a clinical diagnosis. The collection of 37 human sera is listed in Table 5.1. Pooled sera containing individual serum from group (CD, NCGS and HCBD) were prepared adding the same amount of serum from the three types, respectively.

5.2.3. mTG Treatment of Flour Dough

In order to induce protein cross-link in dough samples, the experiment was carried out using 1U of enzyme/100 mg flour. Doughs were incubated at 40 °C for 90 min with constant stirring. After incubation, doughs were stored at -20 °C in order to deactivate the enzyme or immediately processed for the protein extraction.

1.1.1. Protein Extraction and Dialysis

Proteins from flour dough, treated with microbial transglutaminase, were extracted with different buffers in order to obtain extracts containing different protein composition: total protein extract (TE) and three enriched fractions, albumins and globulins (F1), prolamins (F2) and glutelins (F3). The ratio of extraction was 1 g of flour/10 mL of buffer. All operations were carried out at 4 °C. To prepare total protein extract, the dough was suspended in 100 mM Tris HCl pH 8.5; 20% glycerol; 1.7% β-mercaptoethanol. The mixture was sonicated by ultra-sonication for 30 seconds on ice, than incubated o/n with constant stirring. The supernatant was collected after centrifugation at 5000 g for 10 min and then dialyzed

against 0.1 M acetic acid for 24 h using 6-8 kDa cut-off dialysis membrane. The protein enriched fraction was obtained using the same procedure above described but the sequential extraction method was based on the use of different extraction buffers, as described by Rallabhandi and collaborators (Rallabhandi, Sharma, Pereira, & Williams, 2015) with minor modification. First, the dough was extracted twice with 0.5 M NaCl pH 7.5 for 1 h. The two supernatants, containing albumins and globulins were pooled before dialyzing against distilled water. The residual extraction pellet was washed with water for 10 min followed by centrifugation. Then, the pellet was resuspended twice in 70% ethanol for 1 h. The extracts containing prolamins were pooled and the solvent was removed using the speedvac. Finally, the glutelins fraction were extracted by resuspending the residual pellet twice in 50% isopropanol; 1% acetic acid; 0.5% β-mercaptoethanol, followed by dialysis against 0.1 M acetic acid. All the protein extracts were stored at -20 °C until further use. The protein content was estimated using the Bradford method with BSA standards (Bradford, 1976).

ID	Diagnosis	EmA IgA	tTG IgA	DGP-IgG	AGA IgA	AGA IgG
1	CD	pos++-	112.8	na	na	na
2	CD	pos++-	31.6	69.1	7.1	29.3
3	CD	pos+++	132.2	144.3	30.8	151.3
4	CD	pos+--	22.1	68.5	na	na
5	CD	na	139.6	55.6	na	na
6	CD	pos+++	116.9	39.6	3.9	42.7
7	CD	na	135.9	115.9	na	na
8	CD	na	79.4	113.2	na	na
9	CD	pos+++	152.8	101.6	28.5	138.9
10	CD	na	132.8	61.3	na	na
11	CD	pos+++	151.9	106.4	19.1	104.2
12	CD	pos+++	150.9	96.4	49.1	139.6
13	CD	pos++-	105.7	na	na	na
14	CD	na	121.7	17.2	na	na
15	NCGS	neg	4.8	5.1	4.6	40.1
16	NCGS	neg	4.0	9.5	8.8	114.9
17	NCGS	neg	5.4	36.0	12.7	65.1
18	NCGS	neg	5.6	19.7	8.5	34.1
19	NCGS	neg	4.5	7.9	6.0	47.8
20	NCGS	neg	5.3	3.4	8.8	44.4
21	NCGS	neg	5.0	26.0	5.9	85.8
22	NCGS	neg		5.3	8.0	41.6
23	NCGS	neg	5.8	7.6	4.6	51.7
24	NCGS	neg	4.5	4.5	7.3	50.7
25	NCGS	neg	4.5	7.0	7.0	87.0
26	NCGS	neg	5.1	7.6	6.3	40.7
27	NCGS	neg	2.4	4.1	5.1	54.1
28	NCGS	neg	5.7	10.0	8.3	43.0
29	NCGS	neg	5.9	22.8	8.0	119.0
30	NCGS	neg	4.2	3.3	6.6	54.1
31	NCGS	neg	5.2	3.4	7.8	88.5
32	HCBD	neg	1.0	na	na	na
33	HCBD	neg	0.5	na	na	na
34	HCBD	neg	1.5	na	na	na
35	HCBD	neg	1.0	na	na	na
36	HCBD	neg	0.8	na	na	na
37	HCBD	neg	0.5	na	na	na

Table 5.1 Clinical diagnosis and serum antibodies of patients' sera. Coeliac disease (CD), non-celiac gluten sensitivity (NCGS), and healthy control blood donors (HCBD). EMA= anti-endomysial antibodies; tTG= anti-tissue transglutaminase antibodies; DGP=anti-deamidated gliadin peptide antibodies; AGA= anti-gliadin antibodies; IgA= immunoglobulin A; IgG= immunoglobulin G; pos+--= weak positive; pos++-=positive; pos+++=strong positive; neg= negative; na=not available.

5.2.4. Protein Separation by SDS-PAGE

Protein extract composition was examined and compared by SDS-PAGE. Electrophoresis was performed according to the method of Laemmli and the protein extracts were separated on the basis of their molecular weights (Laemmli, 1970). Samples were treated with sample buffer for 5 min at 95 °C and then run on 15% polyacrylamide slab gels. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 at room temperature, and were destained in 10% (v/v) acetic acid. Gels were scanned and analysed in order to measure intensities of specific protein bands using Bio-Rad Image Lab 4.0.1. Software.

5.2.5. In-Gel Digestion of Protein and HPLC-MS Analysis

SDS-PAGE of protein extracts was performed using three extract amounts (10, 30, and 50 μg/lane, respectively). Protein bands were cut and digested with diluted trypsin (8 ng/μL) at 37 °C for 2 hours using the ProteoExtract All-in-One Trypsin Digestion Kit (Millipore). HPLC system (Model UltiMate 3000, Thermo Fisher Scientific) controlled by the Chromeleon Chromatography Data System, version 7.1.0.898 (Thermo Fisher Scientific) was used for high-performance liquid chromatography analysis. In-gel digested peptides were separated on a ply-(styrene-divinylbenzene) separation column (150 mm x 0.2 mm) at 40 °C, flow rate of 0.1 uL/min and a linear gradient of 0-40% acetonitrile in 0.050% aqueous trifluoracetic acid in 30 min. Measurements were performed in positive ionization mode by means of online hyphenation of the HPLC system to a linear ion trap-Orbitrap mass spectrometer (Model LTQ Orbitrap XL, Thermo Fisher Scientific). For peptide identification, the most intense precursor ions was fragmented by Collision-Induced Dissociation (CID) and Xcalibur, version 2.0.7 SP1 (Thermo Fisher Scientific) was

used to control the mass spectrometer and data acquisition. For protein identification, data were blasted against the UniProtKB database using Proteome Discover 1.4 (Thermo Fisher Scientific).

5.2.6. Analysis of the IgG-binding Capacity by ELISA

In order to evaluate structural epitopes of the flour proteins upon microbial transglutaminase treatment, ELISA was performed using sera from sensitive patients. Each well of the maxisorp plate (Nalge Nunc, Rocherster, NY) was coated with 50 µL of the protein sample (5 µg/mL) in phosphate buffered saline (PBS) o/n at 4 °C. The plate was washed twice and subsequently nonspecific binding sites were blocked with 200 µL/well of 1% BSA in tris buffered saline containing 0.05% Tween-20 (TBS-t), for 1 h at room temperature. Then, the IgG-binding capacity was tested by the addition of 50 µL/well of diluted patients' sera or pooled serum, and healthy controls' sera, incubated o/n at 4 °C. For each protein sample, blank controls were tested using 0.5% BSA in TBS-t instead of the diluted serum. After four washes, 50 µL/well alkaline phosphatase-conjugated polyclonal anti-human IgG antibody (Thermo Fisher Scientific), diluted 1:1000 in 0.5% BSA in TBS-t, were added. After 1 h of incubation at 37 °C and another one at 4 °C, the plate was washed four times with TBS-t, and developed with 50 µL/well of substrate solution, consisting of 10 mM p-nitrophenyl phosphate (PNPP) dissolved in alkaline substrate buffer (pH 9.8), composed of 1M diethanolamine and 1mM $MgCl_2$. The absorbance was recorded using a microplate ELISA reader at 405 nm (ref. 490 nm). The IgG-binding capacity of each protein sample was characterized by corresponding OD values. ELISA protocol, coating conditions, reagent dilutions, buffers, and incubation times were tested in preliminary experiments. Several serum dilutions (ranging from 1:250 to

1:2500) were tested in order to identify the optima amount of antibodies for the development of the ELISA analysis.

5.2.7. R5-based Analysis of Wheat Dough Protein

The immune-reactive proteins in wheat dough and sourdough, after enzymatic processing, were qualitatively detected using R5 IgG monoclonal antibody (Operon S.A., Cuarte de Huerva, Spain) raised against gluten-toxic epitopes. The complementary western blot and ELISA techniques were used to determine the gluten content in wheat samples, as the R5 antibody specific for wheat proteins.

After separation by SDS-PAGE, wheat prolamin enriched fractions were electro-transferred to a nitrocellulose membrane for western blot analysis. Antibodies binding to the blots were visualized by staining with alkaline phosphatase-conjugated secondary anti-mouse antibody, using 3-Amino-9-ethylcarbazole (AEC).

The content of gluten toxic peptides of dough and sourdough total protein extracts after processing, has been determined using maxisorp plates for R5-based ELISA analysis.

5.2.8. Statistical Analysis

All data were processed in Office Excel (Microsoft, USA), and reported as mean ± SD, unless otherwise stated. Data were tested using GraphPad Prism by one-way ANOVA. Differences were considered significant when p-values were less than 0.05. A very significant difference was concluded based on a p-value of less than 0.01.

5.3. Results and Discussion

5.3.1. Effect of mTG on Protein Extracts

The microbial transglutaminase protein cross-linking products has been evaluated, after protein extraction and separation by SDS-PAGE. Enzyme activity, at our experimental conditions, was clearly proven by changes in protein pattern. In particular, the presence of HMW protein bands at the top of the resolving gel is a result of the protein aggregation. As shown in Figure 5.1 the changes in protein pattern of gluten and gluten-free total extracts, after microbial transglutaminase treatment, are evidenced by the disappearance in protein bands and the accumulation of protein aggregates that remain at the top or cannot enter the resolving gel. The result of the enzymatic process on dough samples is the modification of proteins through introduction of cross-links in the presence of glutamine residues.
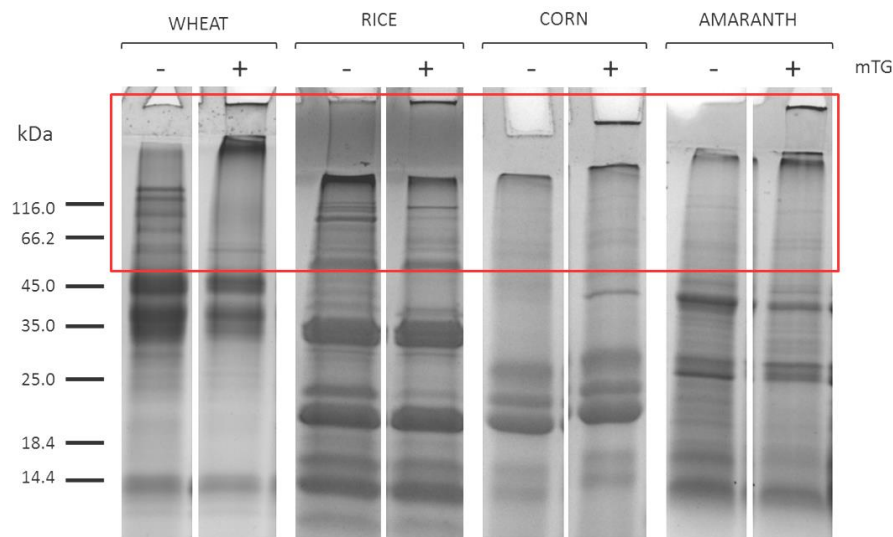
Figure 5.1 SDS-PAGE of total protein extracts from gluten and gluten-free flours before (-) and after (+) microbial transglutaminase (mTG) treatment.

Enzyme effects were evaluated on protein enriched fractions in whole samples. As an example, the rice protein distribution on SDS-PAGE is shown in Figure 5.2.

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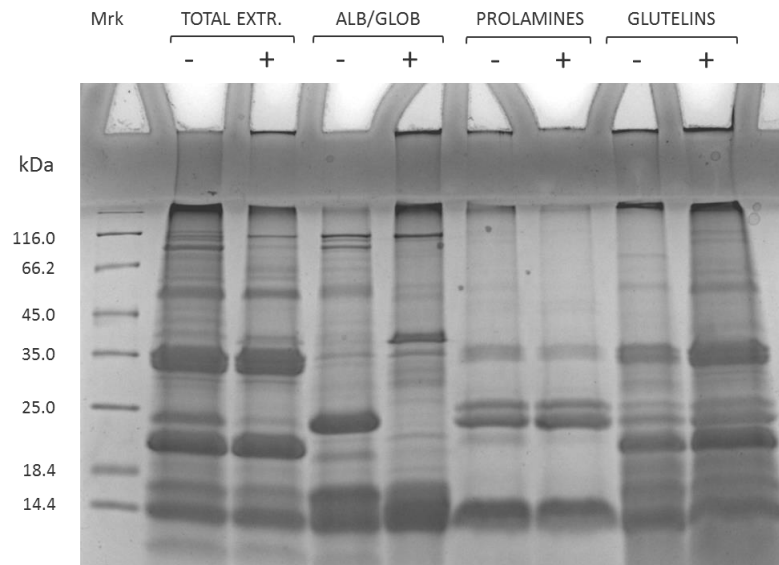
Figure 5.2 SDS-PAGE of total protein extract and enriched protein fractions from rice dough before (-) and after(+) microbial transglutaminase treatment

As suggested by the protein separation, the albumin and globulin (F1), and glutelins (F3) enriched fractions are the main involved in the protein aggregate formation. Similar results were also observed in all gluten and gluten-free flour dough treated (data not shown).

5.3.2. HPLC-MS Analysis of Protein Bands

In order to examine protein aggregates and discover the proteins on which microbial transglutaminase preferentially acts, in gel digestions of protein bands were carried out.

As F1 resulted the fraction most involved in proteins aggregation effects, three increasing amounts of extract were separated by SDS-PAGE in order to clearly observe changes in protein bands, in all samples after enzyme treatment. Figure 5.3. shows the enriched fraction of corn albumin and globulin, separated by SDS-PAGE.
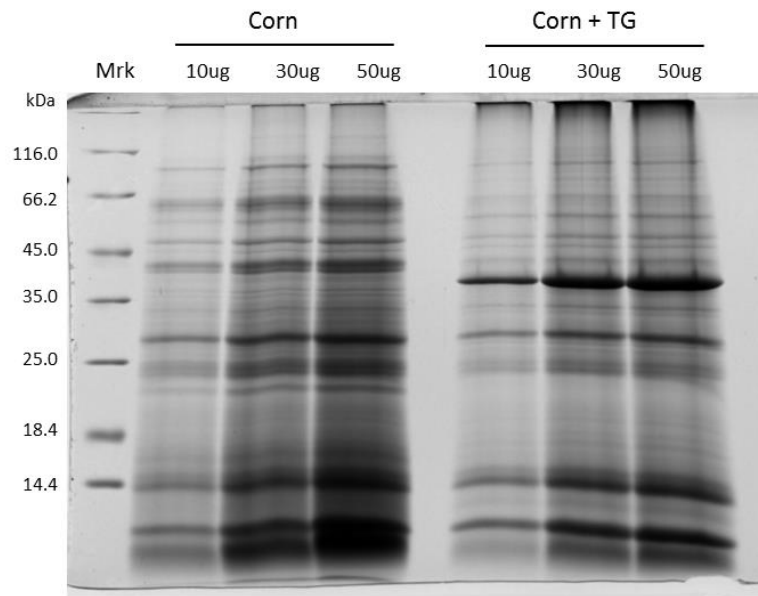
Figure 5.3 SDS-PAGE of three amounts of albumin and globulin (F1) protein fraction from corn dough before and after enzyme treatment.

Protein aggregates at HMW, and principal changed bands along the resolving gel, were cut, and digested peptides were obtained by treatment with trypsin. Peptides separation and detection were carried out using HPLC system and mass spectrometry. The results of protein identification did not allow obtaining clear information about the precursor and structure of the protein aggregates. More specific analyses, such as two dimensional gel electrophoresis and spot analysis, have to be done in order to obtain detailed information about the profile of the proteins involved in the microbial transglutaminase reactions.

5.3.3. Immunoreactivity Tested by ELISA

In order to analyse the immunoreactivity of gluten and gluten-free proteins from dough and sourdough prepared with and without microbial transglutaminase treatment, total protein extracts and three protein enriched fractions were tested by ELISA analysis to assess their IgG-reactivity.

The IgG-binding capacity of sera from gluten sensitive patients and healthy blood donors has been determined using plates coated with the protein extracts, from each samples using ELISA method. As representative of the sera panel used in this study, the patients' IgG reactivity towards wheat proteins is shown in Figure 5.4.
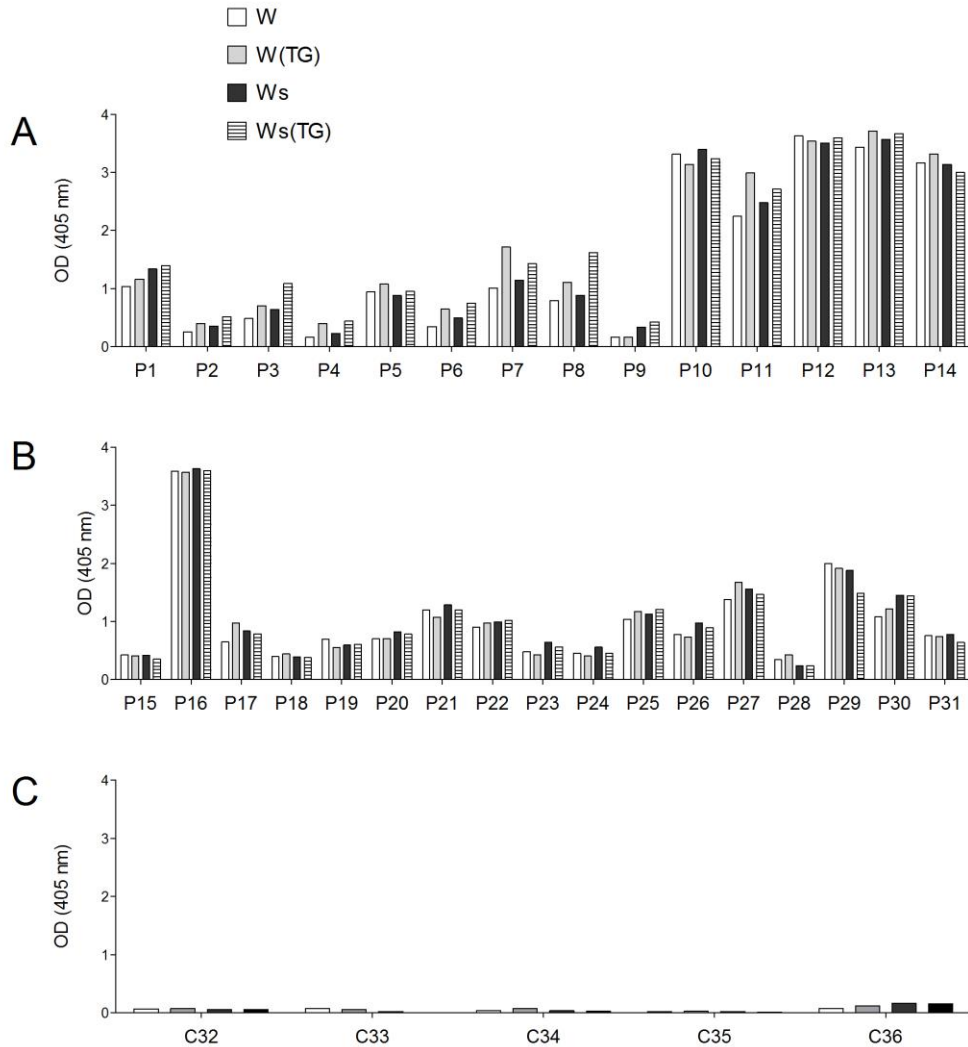

Figure 5.4 IgG reactivity of the sera determined using wheat protein extracts from dough and sourdough treated with microbial transglutaminase (TG), analyzed by ELISA. A: sera from CD patients (from P1 to P14). B: sera from NCGS patients (from P15 to P31). B: sera from HCBD (from C32 to C36).

The IgG reactivity distribution of sera reflects the antibody titers of each individual patient. The highest OD value was reported for the CD patients' sera

(P10-P14) and NCGS P16, whereas no or very low signals were detected in HCBD sera (C32-C36).

Gluten and gluten-free dough protein extracts were separately coated as antigens in microplates and incubated with sera. The IgG-binding capacity of total protein extracts, tested before (-) and after (+) microbial transglutaminase treatment were distributed as reported in Figure 5.5, using pooled sera containing individual serum from group CD patients.
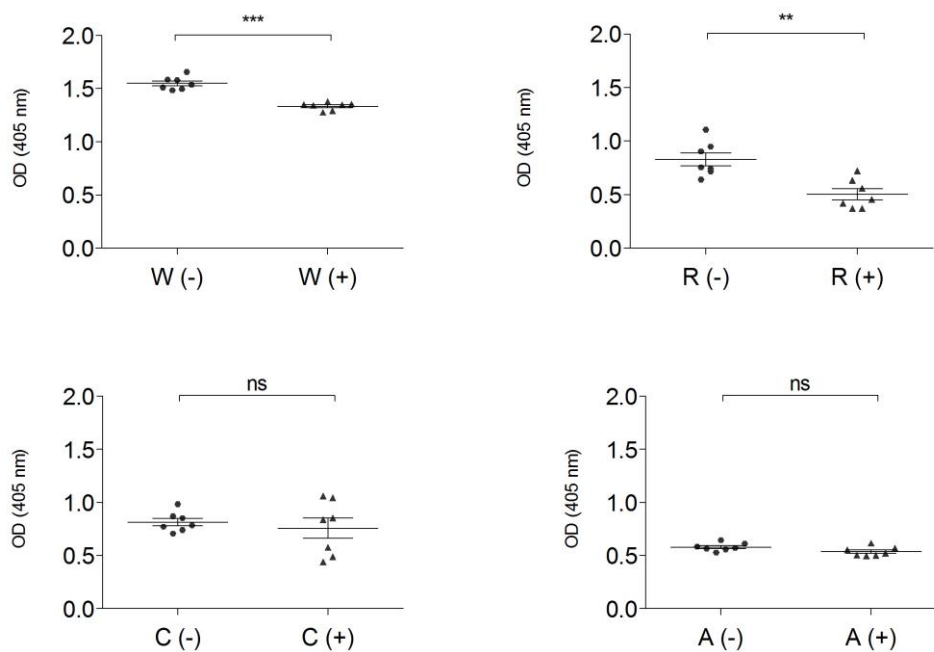

Figure 5.5 IgG-binding capacity, expressed as OD value of gluten and gluten free flours before (-) and after (+) microbial transglutaminase treatment determined by ELISA using pooled CD patient' sera. Data are presented as mean values of 7 replicates with significance level: ns= not significative, *p< 0,05 **p < 0.01, and ***p< 0.001. w: wheat, r: rice, c: corn, a: amaranth.

Both in wheat and gluten free doughs, the immunoreactivity of the protein extracts did not result increase after the enzymatic treatment. In wheat (p value < 0.001) and rice doughs (p value < 0.01), the effect of transglutaminase results in a significant reduction of the IgG-binding capacity using pooled sera from CD patients.

In order to evaluate the effect of microbial transglutaminase and sourdough on gluten-free doughs protein components, the same analysis was performed using a pool of sera from each patient type (CD, NCGS, and HCBD). Different effects of the biological treatment could be the influence on the immunoreactivity of the resulting protein product (data not shown).

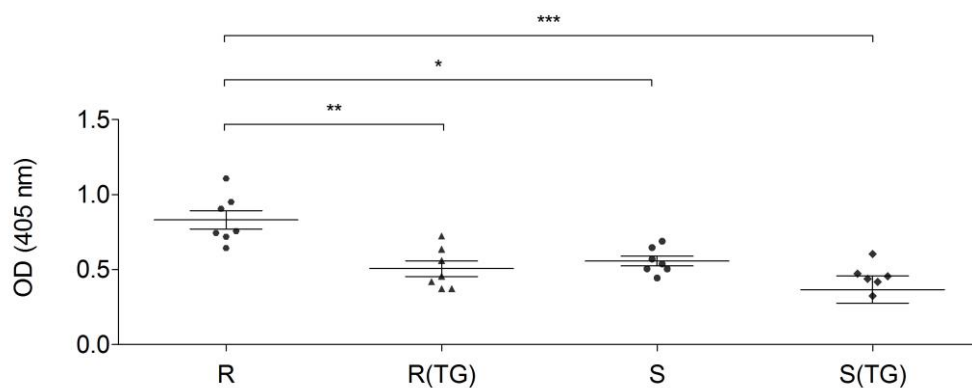As representative data, rice dough total protein extract analysis using a pool of sera from CD patients is shown in Figure 5.6.

Figure 5.6 IgG-binding capacity of rice total protein extracts using a pool of sera from CD patients. The protein from rice dough and sourdough were treated with microbial transglutaminase. Data are reported as absorbance at 405 nm of sera diluted 1:250. Data are presented as mean values with significance level: $*p < 0,05 ** p < 0.01$, and $***p < 0.001$.

The effect of microbial transglutaminase on protein immunoreactivity of rice dough determine a significant reduction (p value < 0.01) of specific antibody binding capacity of the protein, when compared to the control without enzyme treatment. The sourdough fermentation determine a further reduction (p value < 0.05) of the immunereaction, when compared to the control dough without fermentation agent. Finally, the combined use of microbial transglutaminase and sourdough on rice flour results in a significant reduction (p value < 0.001) effect of specific protein epitopes recognized by CD patients' sera.

The IgG-binding capacity of total protein extracts measured using pooled sera from NCGS patients is shown in Figure 5.7.
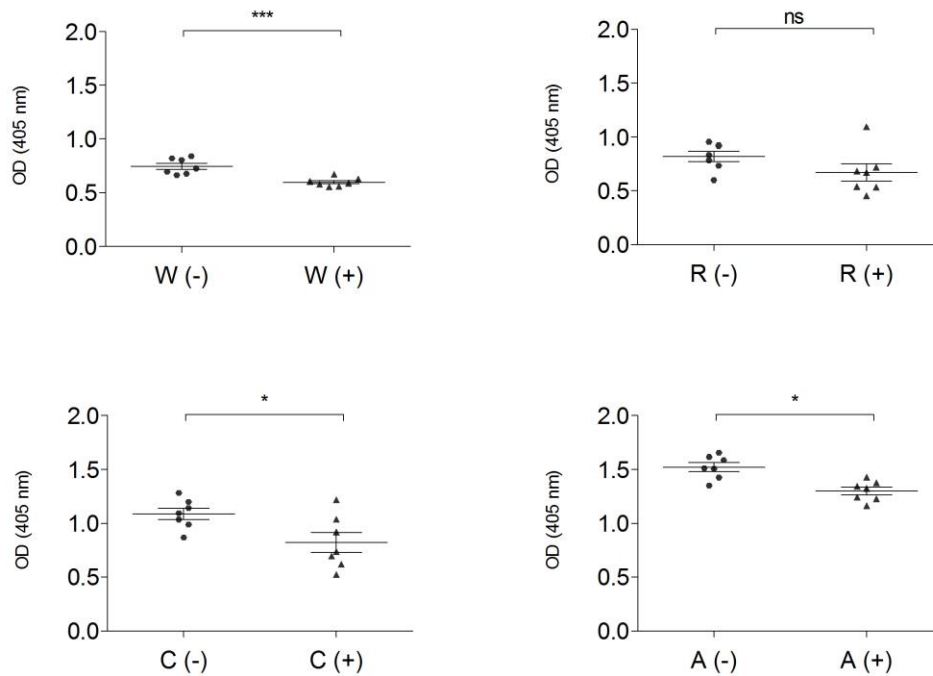

Figure 5.7 IgG-binding capacity, expressed as OD value of gluten and gluten free flours before (-) and after (+) microbial transglutaminase treatment, determined by ELISA using pooled sera from NCGS patients. Data are presented as mean values of 7 replicates with significance level: ns= not significative, *p< 0,05 ** p < 0.01, and ***p< 0.001. w: wheat, r: rice, c: corn, a: amaranth.

Also for this analysis, both in wheat and gluten free doughs, the protein immunoreactivity did not result increase after the enzyme treatment. In wheat dough, the decrease of binding capacity, after the treatment with microbial transglutaminase results in a significance (p value < 0.001) reduction of the protein epitopes responsible of the immune-recognition. Also in gluten-free doughs, such as corn and amaranth the effect of transglutaminase results in a significant reduction of the IgG-binding capacity using sera from NCGS patients (Figure 5.7).

To further evaluate the IgG reactive proteins, dough proteins were extracted by a sequential method using specific buffer solutions. Data distribution of IgG antibody responses, divided by protein fractions are summarized in Figure 5.8.
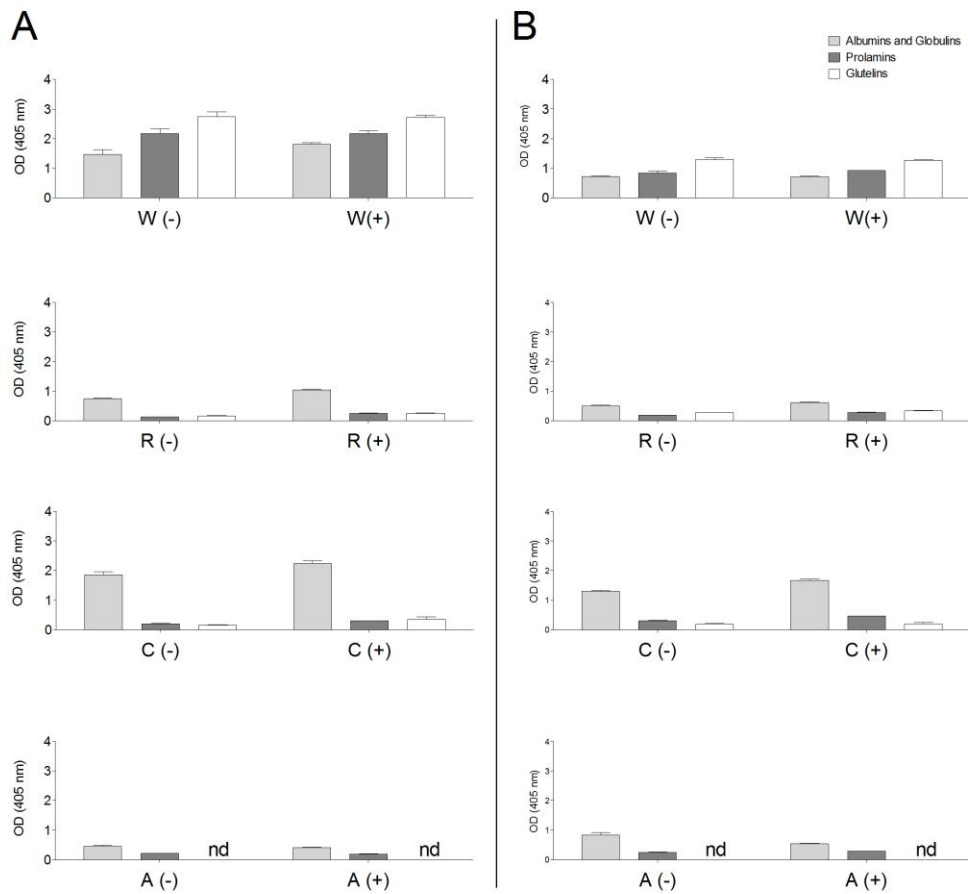
Figure 5.8 IgG-binding capacity, expressed as OD value of gluten and gluten free flours before (-) and after (+) microbial transglutaminase treatment, determined by ELISA using CD (A) and NCGS (B) patient' sera. Three protein enriched fractions were analyzed, albumins and globulins, prolamins and glutelins. Data are presented as mean values ± SD. w: wheat, r: rice, c: corn, a: amaranth.

Data show the different immunoreactivity of the gluten dough compare to the gluten-free samples. In fact, the OD value of the highest wheat protein extracts (prolamins) reaches values of absorbance is around 3, whereas the highest gluten-free value is around 2 for corn albumins and globulins enriched fraction (Figure 5.8 A). In all samples, OD values obtained using CD patients' sera was higher when compared to the corresponding OD value obtained using NCGS patients' sera (Figure 5.8 B). As expected, in wheat dough the immunoreactivity is higher in the prolamins fraction both using CD and NCGS patient' sera. In gluten-free doughs, the immunoreactivity is almost exclusively associated with the albumins and globulins enriched protein

fraction (Figure 5.8 A and B). Moreover, the transglutaminase treatment did not affect the IgG reactivity profile in any of the tested flours.

5.3.4. R5-based Analysis of Gluten Protein

In order to elucidate the catalytic effect of microbial transglutaminase and sourdough on wheat toxic peptide, R5 monoclonal antibody was tested by ELISA and Western Blot analysis. The immunological test based on the R5 monoclonal antibody recognizing potential coeliac-toxic epitopes, which occur repeatedly in gliadins (the prolamin proteins from wheat).

Data from R5-based ELISA analysis of dough wheat total protein extracts before and after processing with microbial transglutaminase and sourdough are reported in Figure 5.9.
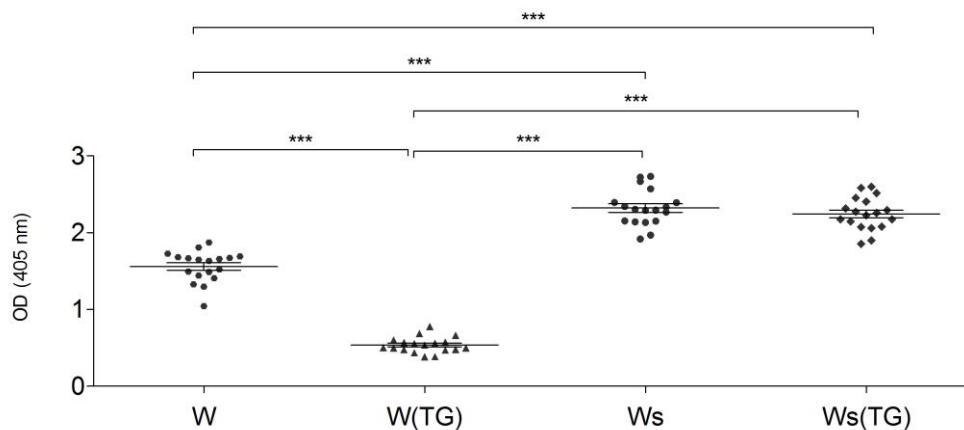

Figure 5.9 R5-based ELISA analysis of wheat total protein extracts before and after processing with microbial transglutaminase and fermentation. Data are presented as mean values of 18 replicates with significance level: *p< 0,05 ** p < 0.01, and ***p< 0.001.

Statistical analysis of the data reported that the addiction of microbial transglutaminase on wheat dough determined a significant (p value < 0.001) reduction of exposed toxic peptide, recognized by R5 antibody. The protein aggregation effect of microbial transglutaminase could determine protein structural changes which affected the quality and conformation of native gluten proteins. The

fermentation process by lactic acid bacteria and yeast, reveal an increase of the OD value, when compared to the control dough probably for the effect of microbial enzymes which degrade the protein substrate determining a major exposition of the gluten toxic peptides. In sourdough, the effect of microbial transglutaminase did not alter the protein profile recognized by R5 (Figure 5.9).

To understand the effect of microbial transglutaminase and sourdough fermentation on gluten proteins, the immunological identification of gluten peptides was performed also by western blot analysis of the prolamin fraction (Figure 5.10).

Wheat dough was treated with two amount of microbial transglutaminase (0.5 and 1 U), with and without microbial fermentation in order to obtain sourdough. Sequential protein extraction was performed, and prolamin fraction was separated and blotted for the immunological analysis (Figure 5.10). As expected band patterns were characterized with strong bands in the molecular weight range of 30-45 kDa.
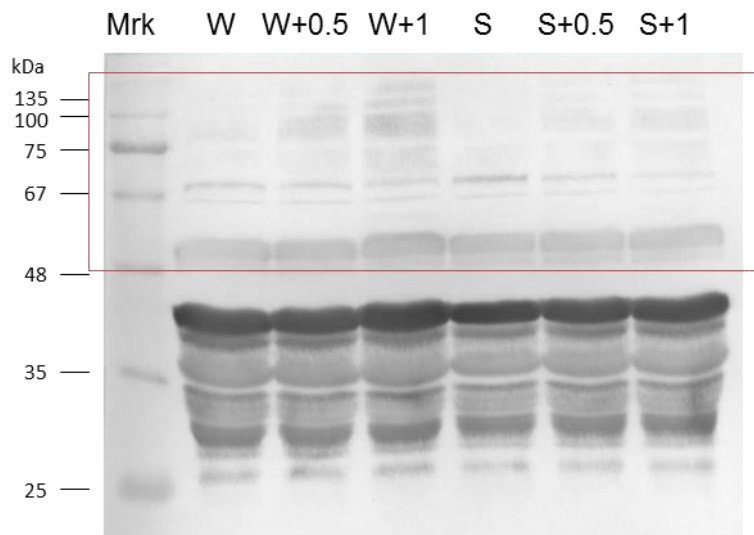

Figure 5.10 R5-based Western Blot of wheat prolamins enriched fraction. mrk: molecular weight marker; w= prolamins extract from wheat dough; w+0.5=prolamins extract from wheat dough treated with 0.5 U of transglutaminase; w+1=prolamins extract from wheat dough treated with 1 U of transglutaminase; s=prolamins extract from wheat sourdough; s+0.5=prolamins extract from wheat sourdough treated with 0.5 U of transglutaminase; s+1=prolamins extract from wheat sourdough treated with 1 U of transglutaminase.

Some differences about immuno-detection of bands are visible at the HMW region (Figure 5.10, in evidence). In particular, bands' intensity result increased when transglutaminase was added, in a dose dependent manner. The corresponding bands were lower evidenced in sourdough (Figure 5.10, S, and S+ transglutaminase units). From this analysis the combination of microbial transglutaminase and sourdough resulted in a reduction of patterns and intensity levels of bands corresponding to some gluten protein containing toxic peptides at HMW (red line).

The capacity of lactic acid bacteria to reduce the level of immunogenic peptides content in sourdough has been studied by several authors. In particular, the manufacturing of wheat and rye breads or durum wheat pasta by using selected lactic acid bacteria sourdough markedly decreases the toxicity of prolamin epitopes even if the concentration of gluten in these foods remained above 6000 ppm (De Angelis, et al., 2006; Di Cagno, et al., 2005; Di Cagno, et al., 2002; Gobbetti, Di Cagno, & De Angelis, 2010). The use of a mixture of ten selected lactic acid bacteria together with fungal proteases, in bread making, and traditional long-time fermentation (48 hours) under semi-liquid conditions was highly efficient in degrading gluten at a concentration below 20 ppm (Rizzello, et al., 2007) that represents the lowest limit to define a gluten-free matrix. In this study, we performed a 21 hours fermentation process that was able to slightly decrease gluten toxic peptides, immune recognized by the monoclonal R5 antibody.

Studying the impact of processing on food structure and allergenic properties is inevitably difficult because the nature of the food matrix is very complex and subjected to not yet clarified molecular interactions.

On the basis of our results, we consider the immunological analysis, based on the use of gluten sensitive patient's sera and R5 antibody, as a tool for the immune

characterization of proteins from dough samples in terms of epitopes changes. Enzyme cross-linking effects did not apparently affect antibody binding capacity, in our experimental condition. In literature other authors reported similar considerations about immunoreactivity results on cross-linked processed food. Ruh, et al. (2014) have studied the immunoreactivity of pasta dough treated with microbial transglutaminase using CD patients' sera. They observed no immunological changes of the gliadin extracts (Ruh, Ohsam, Pasternack, Yokoyama, Kumazawa, & Hils, 2014), while, Cabrera-Chàvez, et al. (2008) reported an increased immunoreactivity of a CD serum pool to gliadin from bread treated with microbial transglutaminase (Cabrera-Chávez, Rouzaud-Sández, Sotelo-Cruz, & Calderón de la Barca, 2008).

In summary, treatment of gluten-free flour doughs with microbial transglutaminase leads to an increase in cross-links and reorganization of protein substrate. This does however not lead to significant changes in immunoreactivity of the protein extracts for both celiac disease and non-celiac gluten sensitive patient's sera.

5.4. Conclusions

The replacement of gluten in bread dough is one of the biggest challenges when working with gluten-free cereal products. This is reflected in the evaluation of products currently on the market which are mainly of lower quality. The wheat protein, namely gluten, has such a wide variety of tasks in bread making, so that without it, a wide range of ingredients are needed to achieve a good quality product. Transglutaminase is being used to improve the functional properties of poor flour proteins as a food ingredient. It is responsible to induce protein structural modifications that improve the functional properties of the final product.

In this study, a wide range of gluten-free cereal dough and sourdough were collected and subjected to enzymatic treatment using microbial transglutaminase, from *Streptoverticillum mobaraense*, in order to obtain improved texture doughs due to protein cross-linking effect. However, they can still threaten the health of a coeliac patient, due to the enzymatic effect on the proteins pattern that may still cause a reaction in gluten sensitive patients.

The molecular approach that was used in this research had the objective to investigate the capacity of microbial transglutaminase to modify protein substrate of flour dough. IgG immunoreactivity of the reaction protein products was investigated in order to evaluate potential epitope changes. Results showed that the application of microbial transglutaminase as an ingredient for gluten-free systems affects the microstructure of the dough, by promoting protein networks. Moreover, no significant increase of IgG-binding capacity using gluten sensitive patients' sera was observed after the enzymatic treatment both in gluten and gluten-free flours. These preliminary results give a perspective in the gluten-free research and suggest their possible use to create innovative products.

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Final Remarks

In this work, the synergistic effects of microbial transglutaminase and a selected consortium of *L. sanfranciscensis* and *C. milleri* on gluten and gluten-free dough characteristics and in bread-making are described.

Transglutaminase has been studied for its ability to organize and create protein networks allowing an improvement of the structure and texture of flour. The microbial consortium has been selected for its capacity to produce interesting metabolites which leads effect on rheological, aroma profile and shelf-life features of the product.

In this thesis is explained what are the effects of the two biological agents, individually and in combination on the protein texture of the dough and also on the structural and aromatic features of baked final product.

In particular, results showed that the properties of flour proteins, in terms of dough protein structure, could be successfully improved by the action of microbial transglutaminase that promoted the formation of protein networks. This enzyme activity on protein substrates allow to implement dough technological characteristics

giving product's structure-building and improving leavening and gas retention. The protein network obtained in dough after cross-linking reaction, can compensate the lack of gluten in gluten-free dough, improving chemical interactions that lead effects on protein microstructural net. However, in presence of sourdough the degradation of wheat gluten, that occurs during fermentation, reduce the viscoelastic properties, responsible for the leavening capacity of bread. In this context the effect of transglutaminase on protein composition of the dough results in implemented products. In fact, the degradative action of sourdough on protein substrates, which reduces the viscoelastic properties of bread, was compensated by the protein-aggregating effect of transglutaminase. Furthermore, enzyme activity of transglutaminase, in terms of isopeptide bonds formation was increased in presence of sourdough, which allows greater access by transglutaminase enzyme on protein substrate. It has been determined a rise effect of protein reticulating on dough when transglutaminase and sourdough act in combination.

The main benefits deriving from the use of the sourdough were enriched in flavors, leavening and increased in shelf-life. Moreover, it is well known that sourdough contributes to the quality of baked goods as it is rich in nutrients and other compounds with beneficial health effects. Consequently, the interesting volatile compound fingerprints obtained in this study could also be the result of changes induced in protein network by transglutaminase.

Under our experimental conditions, wheat sourdough increased the accumulation of some molecules, such as ketones, MCFAs, and alcohols. This results in a significant enhancement of aroma profiles and interesting effect on the shelf-life of the final product.

In fact, the two biological factors enhanced the formation and accumulation of volatile compounds that are interesting for leavening, aroma and also preservation of bakery products affecting its organoleptic properties.

In our conditions, the application of microbial transglutaminase as an ingredient for gluten-free systems affects the microstructure of the dough, without effects on the allergenicity of the protein components. No-changes in specific epitopes has been revealed after the enzymatic treatment, using gluten sensitive patients' sera.

All this preliminary results suggested the possibility to create innovative products with improved characteristics using this new biotechnological tools. But, further studies are required to fully understand the modifications brought by sourdough and microbial transglutaminase on flour proteins. However, these data highlight the useful and safe combination of sourdough and microbial transglutaminase.

List of Chemical Compounds

Chemical compounds	PubChem CID
1-propanol	1031
2-mercaptoethanol	1567
3-amino-9-ethylcarbazole	8588
4-methyl-2-pentanol	7910
acetic acid	176
acetonitrile	6342
acrylamide	6579
bicinchoninic acid	71068
bicinchoninic acid	71068
biotin cadaverine	49850363
biotinyl cadaverine	83906
chloramphenicol	5959
coomassie brilliant blue R	61365
cycloheximide	6197

Chemical compounds	PubChem CID
cystamine	2915
diethanolamine	8113
dimethylcasein	22978807
ethanol	702
glycerol	753
isopropanol	**3776**
magnesium chloride	5360315
methanol	887
peroxidase	86062779
p-nitrophenyl phosphate	2723710
sodium chloride	5234
sodium dodecyl sulphate	3423265
sodium hydroxide	14798
trifluoracetic acid	6422
Tween 20	443314

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List of Publications

Original Articles

Scarnato, L., Serrazanetti, D. I., Aloisi, I., Montanari, C., Lanciotti, R., & Del Duca, S. *Combination of Transglutaminase and Sourdough on Gluten-Free Flours to Improve Dough Structure* (2016). Amino Acids, 48(10), 2453-2465. DOI 10.1007/s00726-016-2258-4

Scarnato, L., Montanari, C., Serrazanetti, D. I., Aloisi, I., Balestra, F., Del Duca, S. & Lanciotti, R., *New Bread Formulation with Improved Rheological Properties and Longer Shelf-Life by the Combined Use of Transglutaminase and Sourdough* (2017) LWT-Food Science and Technology. DOI: 10.1016/j.lwt.2017.03.042

National and International Congress

[POSTER] 14th Naples Workshop on Bioactive Peptides – The Renaissance era of Peptides in Drug Discovery CIRPEB, Napoli, 12-14 Giugno 2014. *The BIORICE European project: BIO technology for the recovery of valuable peptides from industrial RICE by-products and production of added value ingredients for nutraceuticals, functional foods and cosmetics.* (Scarnato, L., Ferri, M., Tassoni, A.)

[ORAL PRESENTATION] Riunione dei gruppi "Biologia Cellulare e Molecolare" e "Biotecnologie e Differenziamento", Società Botanica Italiana (SBI), Roma, 10-12 Giugno 2015. *Biotechnological treatment for the bakery to increase dough structure, shelf-life and sensory properties.* (Scarnato, L., Aloisi, I., Montanari, C., Balestra, F., Lanciotti, R., Del Duca, S.)

[POSTER] 110° Congresso SBI Onlus, Not only food: sustainable development, agro-biodiversity conservation & human well-being, Pavia, 14-18 Settembre 2015. *Gluten free flour doughs improvement of texture by protein cross-links occurring via microbial transglutaminase from Streptoverticillum mobaraense.* (Scarnato, L., Aloisi, I., Montanari, C., Lanciotti, R., Del Duca, S.)

[POSTER] 3rd International conference on microbial diversity. The challenge of complexity, Perugia, 27-29 Ottobre 2015. *Biotechnological treatment for the bakery to increase dough structure, shelf-life and sensory properties.* (Scarnato, L., Aloisi, I., Montanari, C., Balestra, F., Lanciotti, R., Del Duca, S.)

[POSTER] 111° Congresso della Società Botanica Italiana onlus, III International Plant Science Conference (IPSC), Rome, 21-23 Settembre 2016. *Effect of microbial transglutaminase on gluten free flour proteins.* (Scarnato, L., Gadermaier, G., Ferreira, F., Del Duca, S.)

[POSTER] 109th International Congress of "Società Botanica Italiana onlus" - International Plant Science Conference (IPSC) from Nature to Tecnological Exploitations, Firenze, 2-4 Settembre 2014. *The BIORICE European project: BIO technology for the recovery of valuable peptides from industrial RICE by-products and production of added value ingredients for nutraceuticals, functional foods and cosmetics.* (Tassoni, A., Scarnato, L., Ferri, M.)

[POSTER] 110° Congresso SBI Onlus, Not only food: sustainable development, agro-biodiversity conservation & human well-being, Pavia, 14-18 Settembre 2015. *Effect of salinity on seeds protein composition of three landraces of chenopodium quinoa.* (Aloisi, I., Scarnato, L., Ruiz, K., Biondi, S.)

[ABSTRACT] 4th International Conference on Polyamines: Biochemical, Physiological and Clinical perspectives, Roma, 4-9 Settembre 2016. *Gluten free flour doughs improvement of texture by protein cross-links occurring via microbial transglutaminase from Streptoverticillium mobaraense.* (Del Duca, S., Scarnato, L., Serrazanetti, D. I., Lanciotti, R.)

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