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Influence of lactic acid bacteria activity on redox status and proteolysis in gluten-free doughs

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Abbreviations

AA	amino acid/s
ANOVA	analysis of variance
BB	batter or bread made adding buckwheat pre-dough and buckwheat flour
BR	batter or bread made adding buckwheat pre-dough and brown rice flour
CA	chemically acidified doughs
CFU	colony forming units
DO	dissolved oxygen
DY	dough yield
E.	*Enterococcus*
E-64	proteinase inhibitor E 64
EDTA	ethylenediaminetetraacetic acid
E_h	ORP referred to the SHE
E_{h7}	ORP referred to the SHE at pH 7
EPS	exopolysaccharide
f. hetero	facultative heterofermentative LAB
FAA	free amino acid/s
FAN	free amino nitrogen
G*	complex modulus
GF	gluten-free
GSH	reduced glutathione
GSSG	oxidized glutathione
hetero	heterofermentative LAB
homo	homofermentative LAB
HPLC	High-performance liquid chromatography
L.	*Lactobacillus*
LAB	lactic acid bacteria

MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
MANOVA	multivariate analysis of variance
mV	millivolt
O^r_m	maximal oxygen reduction
ORP	oxidation-reduction potential
P.	*Pediococcus*
PCA	principal component analysis
PepA	pepstatin A
PMSF	phenylmethylsulfonyl fluoride
R	software for statistical analysis
RP-HPLC	Reversed phase HPLC
SD	sourdough
SDS	sodium dodecyl sulfate
SH	thiol
SHE	standard hydrogen electrode
TPA	texture profile analysis
TTA	titratable acidity
V^a_m	maximal acidification rate
V^r_m	maximal reduction rate
W.	*Weissella*
ω	angular frequency

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1 Introduction

Celiac disease (CD) is an immune-mediated disease, which affects approximately 1% of the population in the western world (Arendt et al., 2011; Catassi and Fasano, 2008; Fasano and Catassi, 2012). Normally, CD is triggered by the ingestion of prolamine peptides, which are present in wheat, barley, rye and in oat as well (Sadiq Butt et al., 2008; Wieser and Koehler, 2008). Actually, the only solution for those people to avoid health problems is to follow a gluten-free (GF) diet (Zannini et al., 2012).

Nowadays, the big issue of GF products, especially for bakery products, is that they have a low quality compared to standard wheat and rye products (Arendt et al., 2008a; Hager et al., 2012a, 2012b; Moroni et al., 2009; Zannini et al., 2012). Indeed, GF breads mainly show poor crumb and crust characteristics and low mouth feel and flavor (Gallagher et al., 2004). Moreover, a GF diet might lead to nutritional deficiencies of vitamins, minerals, and fibers. Additionally, another big problem for consumers is the expensive price of those products.

In the last decades, the research for the improvement of GF products was very intensive and several solutions were applied, such as the addition of starch, dairy products, dietary fibers, egg proteins, gum as well as hydrocolloids (Gallagher et al., 2004). Moreover, the use of several GF flours were already tested, e.g., rice, sorghum, oat, buckwheat, amaranth, quinoa and teff (Zannini et al., 2012). Furthermore, even alternative approaches were employed, such as enzymes and high hydrostatic pressure (Renzetti and Arendt, 2009a, 2009b; Renzetti et al., 2012, 2010, 2008a; Vallons et al., 2011). However, the application of such ingredients/additives is partially unsuitable due to the high prices and their allergic reactions. Thus, consumers' demand for natural GF products, having similar quality such as wheat products, is increasing (Zannini et al., 2012). As a consequence, GF sourdoughs are normally applied as natural ingredient to improve the nutritional and technological characteristics (Arendt et al., 2011; Moroni et al., 2009). Indeed, sourdough (SD) can be employed to change the flavor profile of the bread, to decrease the content of antinutrient compounds and to produce antifungal metabolites (Arendt et al., 2011; Koh and Singh, 2008; Moore et al., 2008; Osman, 2004). Moreover, it has been shown that the use of SD can also improve the dough rheology and bread characteristics, such as the volume and staling delay (Moroni et al., 2011b, 2009; Zannini et al., 2012). Furthermore, sourdough containing exopolysaccharides are able to increase the bread volume (Schwab et al., 2008).

One of the used GF flour is buckwheat, a pseudocereal with a high protein content, ca. 12% (Pomeranz, 1983). Moreover, buckwheat shows a high content of essential amino acids,

antioxidants and minerals compared to other GF flours (Hager et al., 2012b; Pomeranz, 1983). Besides, buckwheat flour is also getting interesting due to its functional properties. Indeed, the addition of this flour into mixed formulations can improve the baking performances (Mariotti et al., 2013). According to this knowledge, buckwheat flour could be a very interesting substrate to carry out SD fermentations and to apply them for baking tests.

Even though the research in GF bread was intensive, some fields are not completely discovered, such as redox potential changes and proteolysis during GF sourdough fermentations. Merely, some research works were carried out to investigate the effect of commercial redox and proteolytic enzymes on the GF bread structure (Renzetti and Arendt, 2009a, 2009b; Renzetti et al., 2010). In fact, the synergic effect between redox potential changes and proteolysis is well known in wheat sourdoughs (Gänzle et al., 2008). Moreover, it has been shown that the content of free thiol groups and proteolysis can have an influence on the final bread characteristics (Joye et al., 2009). However, these phenomena were still not investigated during GF sourdough fermentations and furthermore the effect of SD, containing different concentrations of thiols and different proteolysis steps, on the GF bread characteristics was not investigated as well.

Accordingly, this work has different goals, at first to investigate the effect of lactic acid bacteria (LAB) on the extracellular redox potential changes and to monitor LAB whole microbial activity during buckwheat sourdough fermentations. Secondly, to observe the possible synergic effect between LAB reducing activity and proteolysis process. Thirdly, to understand the effect of extracellular redox potential on the microbial activity. Finally, to investigate the effect of different buckwheat SD (with different thiols content or different fermentation time) on GF bread characteristics. Moreover, to understand the working mechanisms of microbial activities, several variables were taken into account and analyzed using descriptive statistics and multivariate analysis. The same approach was applied to understand which variables have an effect on GF bread characteristics.

1.1 Celiac disease

'Celiac disease is a unique autoimmune disorder, unique because the environmental precipitant is known' (Green and Cellier, 2007). CD is caused by the ingestion of gluten that is normally present in wheat, barley and rye. After ingestion, undigested α-gliadin fractions remain in the intestinal lumen. These peptides go through the epithelial intestine's barrier and interact with antigen-presenting cells in the lamina propria (Green and Cellier, 2007). The working mechanism of CD is well described in Fig. 1, which was made up by Green et al. (2007).

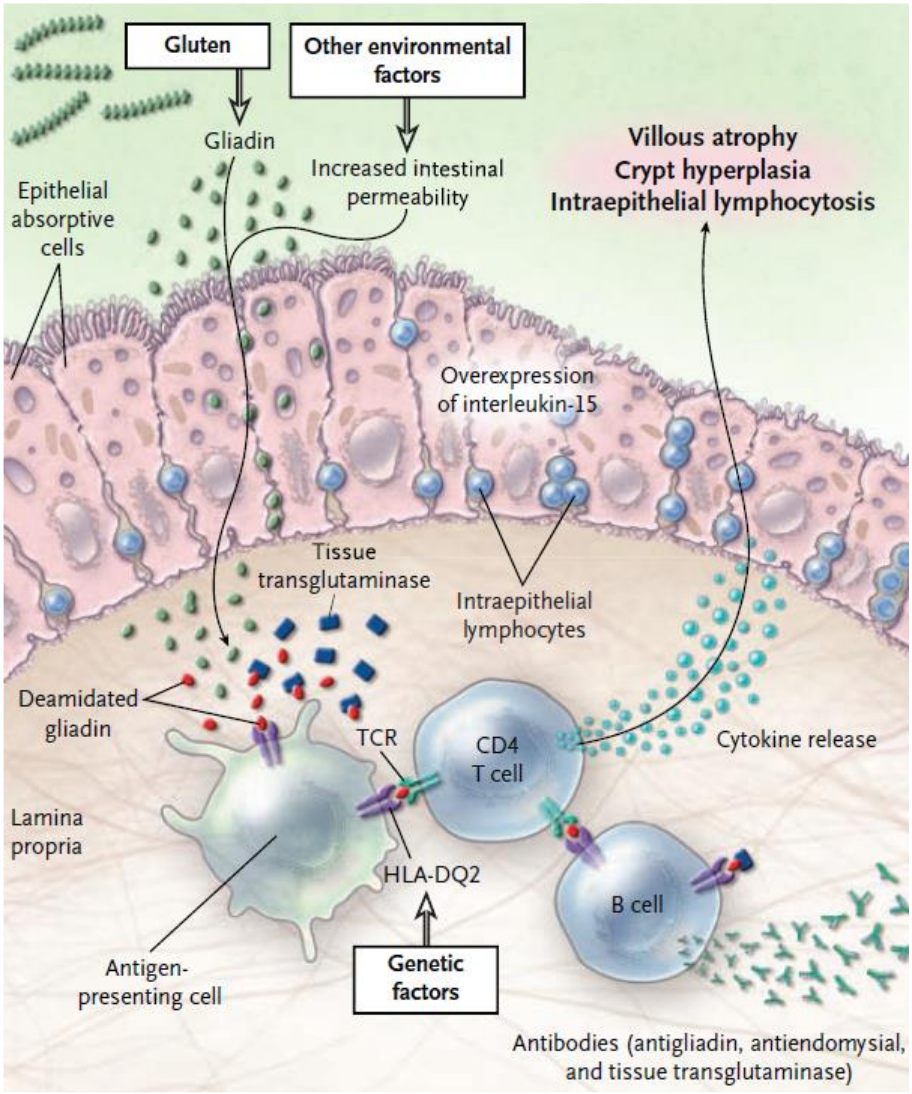

Fig. 1: schema of CD working mechanism (Green and Cellier, 2007). To have a deep understanding of the abbreviated terms and mechanism, the reading of the related paper is required.

To date, CD interests 0.6 to 1.0% of the world population, while wide differences were observed in some European countries, such Germany (0.3% of the population) and Finland

(2.4% of the population) (Fasano and Catassi, 2012). Moreover CD affects both children and adult patients (Fasano and Catassi, 2012; Fasano et al., 2013).

1.2 Gluten-free flours

GF flours originate from the milling process of cereal (teff, sorghum, rice, maize) and pseudocereal grains (quinoa, buckwheat and amaranth) (Alvarez-Jubete et al., 2009; Hager et al., 2012b). One of the most used GF flour is rice, which is mostly employed in the industry as well as for research purpose. However, it contains ca. 80% of starch and a low protein content, ca.7.3% (Hager et al., 2012b). Moreover, breads baked only with rice flour did not show a good structure (Hager et al., 2012a).

Buckwheat flour is becoming more interesting due to its high nutritional and technological properties (Hager et al., 2012b; Mariotti et al., 2013; Pomeranz, 1983). This flour shows a high protein (ca. 12%), antioxidant and mineral content compared to the other GF flours (Hager et al., 2012b). Moreover, it has been shown that buckwheat flour has also a high content of essential amino acids (Pomeranz, 1983). Actually, a disadvantage of this flour is the bitterness due to the high phenolic content (Hayali et al., 2013). Nonetheless, GF breads baked with buckwheat flour show a better structure compared to other GF breads, except for them baked with oat flour, which are similar characteristics to wheat breads (Hager et al., 2012a). Moreover, it has been shown that buckwheat breads treated with transglutaminase has better characteristics than non-treated breads (Renzetti et al., 2008b). Besides, the use of buckwheat flour into bread mixtures can increase notably the nutritional value as well as the baking performances. Indeed, the addition of 40% buckwheat flour improves the leavening capacity of GF breads. Furthermore, this GF flour has the capacity to increase the dough viscosity due to its high content of dietary fiber and to the gelling properties of its starch (Mariotti et al., 2013). Actually, other researchers have demonstrated the increase of baking performances adding 30% of buckwheat flour into a GF rice bread (Dvořáková et al., 2013).

Overall, buckwheat flour displays very interesting nutritional and technological properties for GF breads. Thus, further investigations on buckwheat SD could be very helpful for the improvement of GF bakery products.

Another widely used GF flour for GF bread formulations is rice (Hager et al., 2012a). This flour is very suitable due to bland taste, good digestibility and white color (Arendt et al., 2008b; Hager et al., 2012a). However, rice flour shows a low protein content (ca. 7.3 %) which displays poor functional properties (Arendt et al., 2008b). Moreover, rice flour

exhibits a high starch content, 78 g/100 g, and 21 % of it is based on amylose. Furthermore, the amylose content can differ over the cultivars depending also on the environmental variation. Nevertheless, changes of amylose content influence the pasting behavior and viscoelastic properties. Thus, to improve the functional properties of rice flour, hydrocolloids are often employed in GF bread formulations based on rice flour (Arendt et al., 2008b; Hager et al., 2012b). Compared to wheat system, the addition of commercial proteases increases the specific volume of rice bread due to low resistance to deformation (Renzetti and Arendt, 2009a). Thus, such results demonstrate how this GF system completely differs from the wheat system where proteolysis shows a negative influence on the final bread quality. On the other hand, enzymes such as glucose oxidase and transglutaminase are able to promote a formation of a protein network, which can increase the specific volume of rice breads (Arendt et al., 2008b).

1.3 Gluten-free breads and sourdough application

Gluten is a protein normally formed during dough mixing process and it is built by depolymerization and re-polymerization reactions (Weegels et al., 1997). Gluten is very important in wheat and rye systems due to its capacity to provide dough viscoelastic and water absorption behaviors (Wieser, 2007). Indeed, the bread making quality is mainly influenced by the ratio of gliadin/glutenin protein fraction (Joye et al., 2009). Moreover, gluten mainly provides the structure of breads, e.g., appearance and crumb structure (Gallagher et al., 2004).

Therefore, the big issue for bakers and food technologists is the replacement of this protein in GF breads. Indeed, these type of bakery products show low structure quality, poor mouthfeel and flavor (Gallagher et al., 2004). Moreover, GF breads are mainly based on starch and therefore they are affected by fast staling activity (Moroni et al., 2009). An overview over GF bread structure is displayed in Fig. 2.

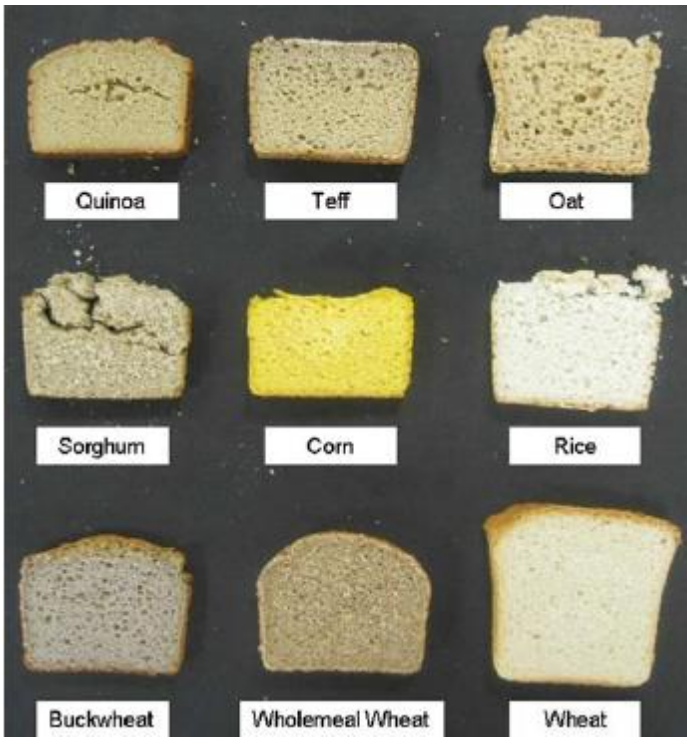

Fig. 2: bread slices of breads baked with quinoa, teff, oat, sorghum, rice and buckwheat flour (Hager et al., 2012a).

However, another issue of GF breads is the low nutritional properties. Indeed, they display low levels of vitamins, minerals, and dietary fibers compared to gluten-containing breads (Moroni et al., 2009; Zannini et al., 2012).

In the last decades, several different ingredients or additives (flour mixtures, dairy products, hydrocolloids, egg proteins, and commercial enzymes) were employed to improve the quality of GF breads. However, with these additions a better quality of the products was reached but this also has some disadvantages, e.g., allergic reactions (egg proteins and lactose) and high prices (Zannini et al., 2012). Due to these reasons and high demand of healthy ingredients from consumers, the most used solution is the SD addition (Arendt and Moroni, 2013). In fact, the addition of SD can increase nutritional properties, leavening capacity as well as crumb structure (Arendt et al., 2011; Moore et al., 2007; Zannini et al., 2012). Nonetheless, another interesting trend is the use of EPS-producing starter culture in GF sourdough fermentation. Indeed, these type of polymers are normally used in the food industry as thickeners, stabilizers, viscosifiers, emulsifiers as well as gelling agents (De Vuyst, 2012). Thus, the application of SD can improve notably the bread characteristics, such as volume, crumb softness and staling delay (Rühmkorf et al., 2012; Schwab et al., 2008; Wolter et al., 2013).

1.4 Redox potential and redox agents in dough system

Gluten is constituted by monomeric gliadins and polymeric glutenins (Belitz et al., 2004). The three-dimensional network of gluten is mainly stabilized by the presence of disulfide bounds. Basically, gliadins, except for ω-gliadins, contain intramolecular disulfide bounds, while glutenins form intermolecular disulfide bounds (Wieser, 2007). Thus, the application of redox agents (molecular oxygen, potassium bromate, ascorbic acid, iodate and glucose oxidase) can influence the quality of the gluten network and consequently the dough kneading process as well as the bread properties (Joye et al., 2009). Moreover, the SH/SS interchange reaction mechanism is well known in wheat dough system (Grosch, 1999; Reinbold et al., 2008).

On the other hand, even LAB can have an influence on the redox status of gluten. Indeed, heterofermentative strains, such as *Lactobacillus (L.) sanfranciscensis*, show enzymatic activities, which are able to influence the redox status of gluten, as also shown in Fig. 3 (Vermeulen et al., 2006). Basically, this activity is supported by specific enzymes, such as glutathione reductase, which is able to reduce oxidized glutathione (Jänsch et al., 2007).

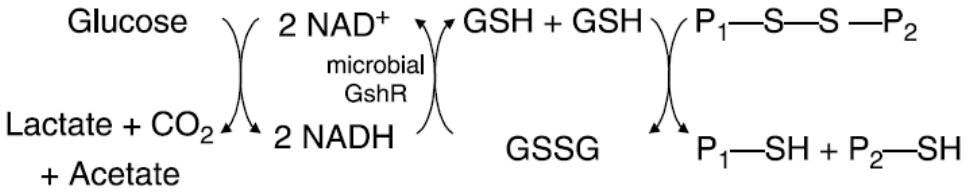

Fig. 3: Redox reaction mechanism between microbial activity of *Lactobacillus sanfranciscensis* and gluten based on glutathione reductase activity (Vermeulen et al., 2006).

Actually, there are only few research works about the role of redox agents in GF systems and the work mechanism is nevertheless not completely understood. It has been demonstrated that the addition of glucose oxidase influences the specific volume and crumb hardness of corn, sorghum and oat bread (Renzetti and Arendt, 2009b; Renzetti et al., 2010). Moreover, Yano (2010) has shown that the addition of high glutathione's concentrations increases the volume of rice bread. However, Yano (2012) has been lately observed that oxidized glutathione (GSSG) also has the same effect as well as reduced glutathione (GSH). However, until now the effect of different starter culture (reducing and oxidizing) on redox status of GF sourdough stays unknown. Moreover, even the effect of reducing and oxidizing GF sourdoughs on GF breads stays still not investigated.

1.5 Proteolysis in sourdough fermentations

The breakdown of cereal proteins is mainly caused by endogenous flour proteases during wheat and rye sourdough fermentations. The most important proteases, involved in proteolytic activity, are aspartic proteinases and carboxypeptidases and both are active under acidic conditions (Gänzle et al., 2008). Moreover, it has been proven that the addition of pepstatin A decreased proteolytic activities in SD fermentation with *L. sanfranciscensis*, demonstrating that aspartic proteases are involved in proteolysis of wheat sourdoughs (Vermeulen et al., 2005). Thus, wheat and rye proteinases support the growth of non-proteolytic LAB. Moreover, it has been demonstrated that heterofermentative LAB, which show glutathione reductase activity, are able to increase the proteolysis process by the reduction of intermolecular disulfide bounds of glutenin macropolymer (Jänsch et al., 2007; Loponen et al., 2008; Thiele et al., 2002). However, homofermentative LAB do not show a higher reducing activity and therefore SD containing this type of starter culture exhibited a lower proteolytic activity (Gänzle et al., 2008).

To date, there are no research studies about the proteolytic activity in GF sourdough fermentations. However, it has been shown that proteolysis, induced by commercial proteases, can have different effects on GF bread structure. In fact, proteolytic enzymes show negative effects on buckwheat and sorghum breads, while they improve the structure of oat and brown rice breads (Renzetti and Arendt, 2009a, 2009b; Renzetti et al., 2010).

Moroni et al. (2011) demonstrated that, after leavening process, proteolytic activity affects the protein fraction of buckwheat batters but it is uncertain if this activity depends on endogenous proteases or not. Actually, there is only information about the presence of proteases in buckwheat seeds, but there is no information about the activity of these enzymes during buckwheat sourdough fermentations. In buckwheat seeds are present the following proteolytic enzymes: metalloproteinases, serine, cysteine, and aspartic proteases (Dunaevsky and Belozersky, 1998, 1989; Timotijevic et al., 2003). The understanding of proteolysis working mechanisms in buckwheat sourdough could be very helpful for the production of high-quality SD, which might influence the quality of GF breads.

1.6 Ecology of gluten-free sourdough

‘Spontaneous ‘sour’ dough fermentation is one of the oldest cereal fermentations known in mankind’ (Decock and Cappelle, 2005). Normally, sourdoughs are classified in three categories (Decock and Cappelle, 2005):

- Type I: the SD is started using a part of a previous fermentation and this is the so-called traditional SD.
- Type II: the fermentation is started using adapted strains and the SD is normally liquid. This type of SD is mainly used in the industry.
- Type III: this a dried SD which is normally used by industrial bakeries.

To date, the microbial activity of LAB in wheat and rye SD is well known and understood. Indeed, it has been demonstrated how interaction mechanisms between LAB and yeasts work and even carbohydrate, peptide and lipid metabolism has been intensively investigated in wheat and rye SD (Cagno et al., 2002; Corsetti et al., 2001; De Vuyst and Vancanneyt, 2007; Gänzle et al., 2007; Gobbetti, 1998; Hammes et al., 2005; Venturi et al., 2012). However, at the beginning of the 2000s, investigations about the behaviors of LAB in GF sourdough fermentations have been started. Indeed, it has been observed that typical wheat and rye starter culture is not suitable for all types of GF sourdoughs, because some strains are substrate specific (Moroni et al., 2010; Vogelmann et al., 2009). Besides, the most frequent strains, isolated from different GF sourdoughs, are: *Pediococcus pentosaceus*, *Lactobacillus sakei*, *Lactobacillus brevis*, *Lactobacillus graminis*, *Lactobacillus paralimentarius*, *Lactobacillus gallinarum*, *Lactobacillus plantarum* and *Lactobacillus fermentum* (Edema and Sanni, 2008; Hüttner et al., 2010; Meroth et al., 2004; Moroni et al., 2011, 2010; Vogelmann et al., 2009).

1.7 Oxidation-reduction potential (ORP) measurements

To date, the effect of LAB growth on ORP changes in sourdough fermentations is still not investigated. The pH value is the most used control parameter for SD technology. However, some studies about ORP measurements were just carried out in food fermentations, e.g., in yoghurt, in cheese, in wine and in sauerkraut (Abraham et al., 2007; Jeanson et al., 2009; Kukec and Wondra, 2002; van Dijk et al., 2000). ORP changes are normally correlated with microbial growth and moreover even with consumption of dissolved oxygen in microbial fermentations (Jacob, 1970; Rödel and Scheuer, 2003; Tengerdy, 1961). This control parameter is very powerful to monitor microbial growth during food fermentations, because it could be used as online monitoring tool to predict contamination or spoilage (Olsen and Pérez-Díaz, 2009). Moreover, LAB strains show typical ORP course in specific medium; thus it could be possible to distinguish a typical redox trend from another one caused by contamination (Brasca et al., 2007). ORP monitoring could be helpful for process control in SD fermentations to improve and control the quality of GF SD. Moreover, changes in redox

status have some effect on the thiol content in cereal fermentations (Jänsch et al., 2007) and this could influence bread quality of SD bread.

The ORP measurement is an interesting tool employed in fermentations. It is becoming more interesting in biotechnology, especially in food fermentations (Caldeo and McSweeney, 2012; Jeanson et al., 2009; Martin et al., 2013, 2011; Olsen and Pérez-Díaz, 2009; Topcu et al., 2008; van Dijk et al., 2000). Besides, its control can influence the intracellular redox balance and consequently microbial metabolic activities. ORP is the ratio of oxidative to reductive substances and it displays the oxidation state of a biological system. Moreover, this fermentation parameter can be influenced by temperature, pH and the concentration of redox agents (Liu et al., 2012; Martin et al., 2013).

1.8 Objectives

The objectives of this thesis were focused first on the LAB activity in sourdough and secondly on the effects of SD addition in bread. In sourdough fermentations, the goals were to monitor and describe the reducing activity of LAB using redox potential measurements in buckwheat sourdough fermentations. Moreover, the correlation between the thiol content and redox potential measurements should be more investigated. Furthermore, it is important to investigate the proteolysis process in buckwheat sourdough fermentations. However, the redox potential of the medium could affect the microbial activity during fermentations, thus it should be investigated using different redox conditions. After the understanding of this mechanisms, a microbial screening with more LAB should be performed to confirm the previously described behaviors in buckwheat SD. Besides, even possible changes in buckwheat protein fraction caused by enzymatic activity should be observed. Finally, the effect of ‘reducing’ and ‘oxidizing’ SD on GF breads and bakery yeast activity should be deeply investigated.

2 Materials and methods

A short overview of the applied methods is showed in Fig. 4.

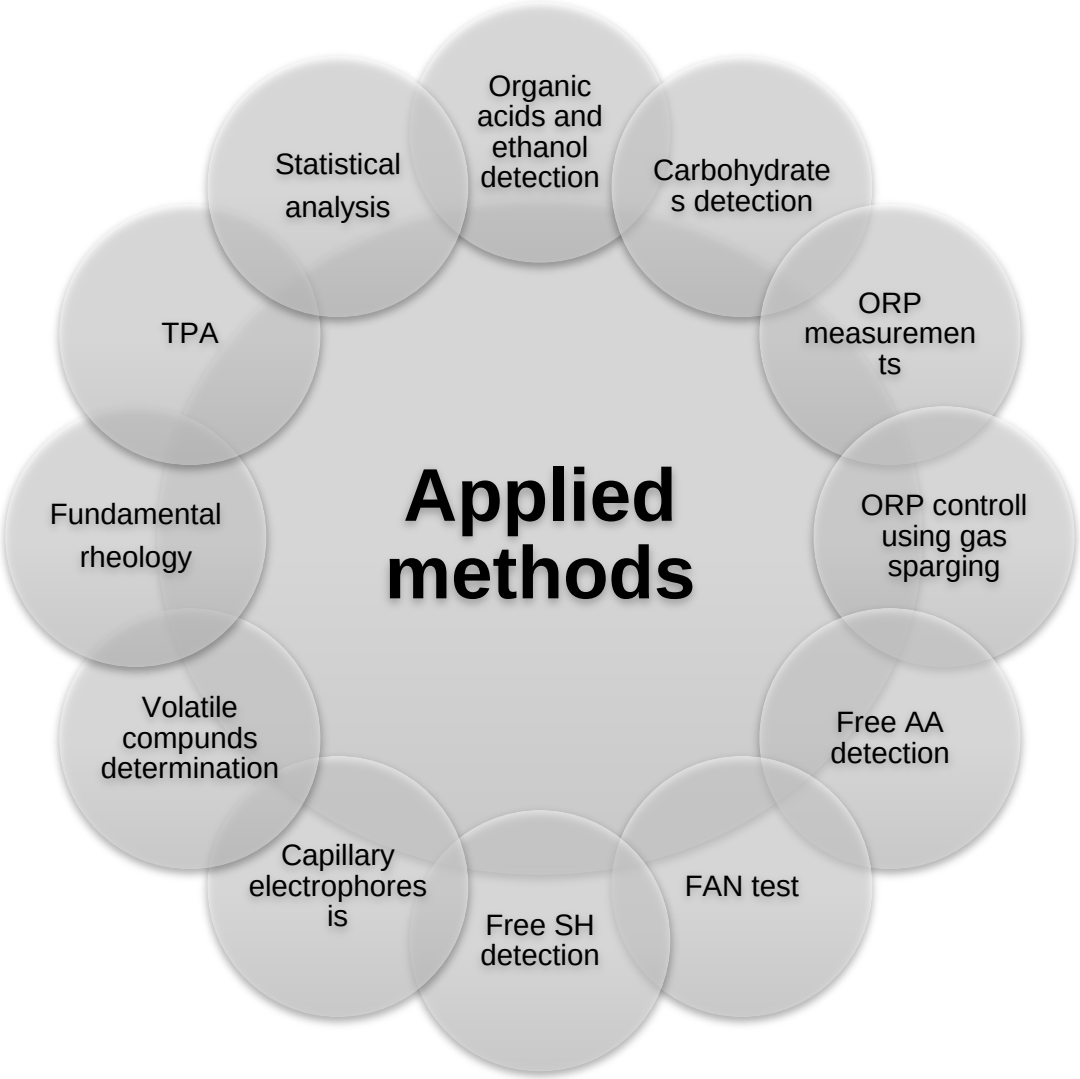

Fig. 4: list of applied methods

2.1 Gluten-free flours

Buckwheat flour was obtained milling hulled organic grains (ZIEGLER & Co. GmbH, Germany) and this type of flour was used for SD fermentations, while buckwheat flour Jade g.f. (Trouw B.V., Netherlands) was used for baking tests. Furthermore, also brown rice flour (Doves Farm Foods Ltd, UK) was employed for baking tests.

2.2 Microorganisms and culture conditions

All the employed strains in this work were obtained from the collection of the chair 'Lehrstuhl für Technische Mikrobiologie' in Weihenstephan (TMW). The strains were cultivated at 30 °C, except for *E. faecalis* which was cultivated at 37 °C in Spicher medium. The formulation for Spicher medium is shown in Table 1.

Table 1: ingredients for Spicher medium preparation

Components	Concentration per liter
Glucose	7 g
Maltose	7 g
Fructose	7 g
Peptone from casein	10 g
Meat extract	2 g
Yeast extract	7 g
Sodium gluconate	2 g
Sodium acetate $3H_2O$	5 g
Diammonium hydrogen citrate	5 g
Potassium dihydrogen phosphate	2.5 g
Magnesium sulfate $7H_2O$	0.2 g
Manganese (II) sulfate H_2O	0.1 g
Iron (II) sulfate $7H_2O$	0.05 g
L-cysteine hydrochloride monohydrate	0.5 g
Tween 80	1 g
Agar for solid media	15 g

The preparatory culture was incubated for 24 h and afterwards the cell pellet was washed twice using a ringer solution (Merck, Germany).

The employed strains are mainly isolated from sourdough and they are listed in Table 2.

Moreover, for the last baking tests, three commercial dry yeasts were employed during batter preparation:

- Pante red (Puratos, Belgium)
- Fermipan rot (Uniferm GmbH)
- Lallemand (Lallemand Inc., Canada)

Further SD fermentations for baking tests were performed using 5 different co-culture:

- LAB1 (*L. mindensis* TMW 1.1206 + *L. brevis* TMW 1.305)
- LAB2 (*L. plantarum* TMW 1.1723 + *L. paracasei* 1.1724)
- LAB3 (*P. pentosaceus* 2.6 + *L. paracasei* 1.1305)
- LAB4 (*L. paracasei* 1.1305 + *L. brevis* 1.1786)
- LAB5 (*P. pentosaceus* TMW 2.6 + *L. mindensis* TMW 1.1206)

Table 2: employed *Lactobacilli* for buckwheat sourdough fermentations

No.	Strains	II abbreviations	Origin	Metabolism
1	*Enterococcus (E.) faecalis* TMW 2.630	E. fa.	sheep cheese	homofermentative
2	*Lactobacillus (L.) brevis* TMW 1.100	L. brev.	sourdough	heterofermentative
3	*Lactobacillus (L.) brevis* TMW 1.1785	L. brev.	rice sourdough	heterofermentative
4	*Lactobacillus (L.) brevis* TMW 1.1786	L. brev.	rice sourdough	heterofermentative
5	*Lactobacillus (L.) brevis* TMW 1.1787	L. brev.	rice sourdough	heterofermentative
6	*Lactobacillus (L.) casei paracasei* TMW 1.1462	L. c. par.	sourdough	facultative heterofermentative
7	*Lactobacillus (L.) graminis* TMW 1.1174	L. gra.	grass silage(DSM 20719)	facultative heterofermentative
8	*Lactobacillus (L.) mindensis* TMW 1.1206	L. min.	sourdough (DSM 14500)	homofermentative
9	*Lactobacillus (L.) paracasei sub. paracasei* TMW 1.1183	L. par. par	DSM 5622	facultative heterofermentative
10	*Lactobacillus (L.) paracasei* TMW 1.1305	L. par.	sourdough	facultative heterofermentative
11	*Lactobacillus (L.) paracasei* TMW 1.1434	L. par.	milk	facultative heterofermentative
12	*Lactobacillus (L.) paracasei* TMW 1.1724	L. par.	sourdough	facultative heterofermentative
13	*Lactobacillus (L.) paracasei* TMW 1.304	L. par.	beer	facultative heterofermentative
14	*Lactobacillus (L.) paralimentarius* TMW 1.1234	L. paralim.	sourdough	facultative heterofermentative
15	*Lactobacillus (L.) paralimentarius* TMW 1.1235	L. paralim.	sourdough	facultative heterofermentative
16	*Lactobacillus (L.) paralimentarius* TMW 1.1726	L. paralim.	rice sourdough	facultative heterofermentative
17	*Lactobacillus (L.) paralimentarius* TMW 1.256	L. paralim.	sourdough (DSM 13238)	facultative heterofermentative
18	*Lactobacillus (L.) plantarum* TMW 1.1204	L. pla	sourdough (DSM 13238)	facultative heterofermentative
19	*Lactobacillus (L.) plantarum* TMW 1.1237	L. pla	sourdough	facultative heterofermentative
20	*Lactobacillus (L.) plantarum* TMW 1.124	L. pla	sourdough	facultative heterofermentative
21	*Lactobacillus (L.) plantarum* TMW 1.1723	L. pla	sourdough	facultative heterofermentative
22	*Lactobacillus (L.) plantarum* TMW 1.460	L. pla	sourdough	facultative heterofermentative
23	*Lactobacillus (L.) plantarum* TMW 1.60	L. pla	sourdough	facultative heterofermentative
24	*Lactobacillus (L.) sakei* TMW 1.1239	L. sak.	sourdough	facultative heterofermentative
25	*Lactobacillus (L.) sakei* TMW 1.22	L. sak.	sourdough	facultative heterofermentative
26	*Lactobacillus (L.) sakei* TMW 1.704	L. sak.	sourdough	facultative heterofermentative
27	*Lactobacillus (L.) sakei* TMW 1.705	L. sak.	sourdough	facultative heterofermentative
28	*Pediococcus (P.) pentosaceus* TMW 2.1036	P. pent.	sourdough	homofermentative
29	*Pediococcus (P.) pentosaceus* TMW 2.6	P. pent.	sake mash (DSM 20333)	homofermentative
30	*Pediococcus (P.) pentosaceus* TMW 2.74	P. pent.	-	homofermentative
31	*Pediococcus (P.) pentosaceus* TMW 2.8	P. pent.	beer yeast (DSM 20336)	homofermentative
32	*Weissella (W.) cibaria* TMW 2.1333	W. cib.	sourdough	heterofermentative
33	*Weissella (W.) confusa* TMW 1.903	W. conf.	sourdough	heterofermentative
34	*Weissella (W.) confusa* TMW 1.918	W. conf.	sourdough	heterofermentative
35	*Weissella (W.) confusa* TMW 1.921	W. conf.	sourdough	heterofermentative

2.3 Sourdough fermentations and dough preparation

Two type of sourdough fermentations were carried out:

- stirred fermentations (600 rpm to avoid phase separation) in 500 mL fermenters BioStat Q (B. Braun-Sartorius, Germany);
- steady state fermentations in plastic beakers and 1.5 mL tubes.

Buckwheat doughs with a dough yield (DY) 350 were prepared using still water (BONAQUA, Germany), while doughs with a DY 500 were prepared for fermentations in 1.5 mL tubes and for inhibitory tests. DY was calculated using the equation 1:

$$\boldsymbol{DY = 100 \times \frac{dough\ weight}{flour\ weight}} \qquad \textbf{Equation 1}$$

Each fermentation was inoculated with a start concentration of ca. 10^7-10^8 CFU/mL dough. Moreover, chemically acidified doughs (CA) were acidified each two hours adding a lactic-acetic acid solution (4:1) and CA containing 5 mM glutathione (CA + GSH) were also prepared in the same way.

2.4 Oxidation-reduction potential (ORP) measurements

ORP was measured using autoclavable redox probes Pt-Ag/AgCl (SCHOTT, Germany) and they were polished using alumina powder 10 µm (Sigma-Aldrich, Germany) (Jacob, 1970). The functionality of redox probes was tested using a standard redox solution (Hanna, Germany).

The ORP, referred to the standard hydrogen electrode (SHE), E_h was calculated using the equation 2.

$$\boldsymbol{E_h = E_m + E_r} \qquad \textbf{Equation 2}$$

E_m is the measured ORP using the redox probe, while E_r is the ORP of the SHE at 30 and 37 °C (204 and 200 mV). Since the pH influences the ORP, the E_h at pH 7 (E_{h7}) was calculated according to the method of Leistern and Mirna (1959):

$$\boldsymbol{E_{h7} = E_h - [(7 - \mathrm{pH})\alpha]} \qquad \textbf{Equation 3}$$

α is the E_h-pH correlation factor (mV/pH unit) it is normally experimentally determined. In our case, the α was 54 mV/pH unit at 30 °C.

2.5 pH and oxygen partial pressure measurements

Dough pH was calculated using autoclavable pH probes (Mettler-Toledo, Switzerland). The probes were calibrated using pH 7 and 4 calibration buffers.

On the other hand, the oxygen partial pressure was measured using pO_2 probes (Ingold, Switzerland) and they were calibrated using N_2 and air.

2.6 ORP control and gas sparging

Extracellular redox potential was held constant by gas sparging at 0.1 L/min with compressed air and forming gas N_2/H_2 (95% nitrogen and 5%). Two different conditions were established, oxidizing with air (high redox potential) and reducing with N_2/H_2 (low redox potential). Fermentations without gas sparging were used as control.

2.7 TTA, pH of dough/batters, strain proteolytic activity and cell counts

The titratable acidity (TTA) of SD was determined according to the method of Meroth et al. (2003), suspending 5 g of SD in 50 mL distilled water. The value of the TTA is displayed as the amount in milliliters of 0.1 M NaOH to reach a final pH of 8.5.

The pH of doughs and batters was measured according to the method of Meroth et al. (2003), adding 5 g of dough/batter in 50 mL distilled water. Afterwards, the solution was stirred and pH measured.

To screen the proteolytic activity of the used strains, calcium caseinate agar (Sigma-Aldrich, Germany) plates were employed, according to the method of Thiele (2003). Colonies, showing a bright ring around, display proteolytic activity.

Cell count was measured as colony forming unit (CFU) per mL dough, due to the liquid consistency of the doughs. Serial dilutions were performed using ringer solution and plated on Spicher agar plates using a spiral plater (Eddy Jet, Germany). Afterwards, the agar plates were incubated at 30 and 37 °C for 1-2 days.

2.8 Verification of sourdough flora

To verify the growth of the used LAB, colony morphology, pH and MALDI-TOF MS measurements were performed. Microbial colonies were analyzed using a Microflex LT MALDI-TOF MS (Bruker Daltonics, Germany). For external mass calibration, a bacterial standard (Bruker Daltonics, Germany) was used. Obtained spectra were analyzed using a MALDI BioTyper 3.0 (Bruker Daltonics, Germany) (Kern et al., 2013).

2.9 Proteases inhibitory tests in buckwheat doughs

In buckwheat seeds, different endogenous proteases are present: metalloproteinases, serine, cysteine and aspartic proteases (Dunaevsky and Belozersky, 1989; Dunaevsky et al., 1998; Timotijevic et al., 2003). To monitor which proteases play a major role in buckwheat dough, several inhibitors with different final concentrations in dough were employed: 200 µM Pep A (Pepstatin A, Applichem, Germany), 1.25 mM PMSF (phenylmethylsulfonyl fluoride, SERVA-Electrophoresis, Germany), 20 µM E-64 (SERVA Electro- phoresis, Germany) and 15 mM EDTA (ethylenediamine tetraacetic acid·Na2-salt, SERVA-Electrophoresis, Germany). The minimal inhibitory concentration was determined as indicated by Jones and Budde (2005).

2.10 Analysis of organic acids, carbohydrates and volatile compounds

Sample preparation for organic acids and ethanol detection was performed adding 500 µL dough with 500 µL distilled water and centrifuged at 15,000 x g for 10 min. To achieve protein precipitation, 400 µL supernatant was mixed with 800 µL distilled water and 10 µL

15 % perchloric acid. Afterwards, it was stored overnight at 4 °C. Finally, the precipitate was eliminated by centrifugation at 15,000 x g for 10 min and the supernatant was used for analysis. Whereas, the sample preparation for carbohydrate analysis was performed adding 500 µL dough with 250 µL 10 % zinc sulfate and 5 mM NaOH. The sample was stored for 20 min at room temperature and then the protein precipitate was eliminated by centrifugation at 15,000 x g for 10 min and the supernatant was analyzed.

Organic acids, ethanol and carbohydrates detection was performed by IEC dual analysis system ICS-5000 (Dionex, USA). Organic acids and ethanol were analyzed using a ReproGel-H 9 lm (Dr. Maisch GmbH, Germany) combined with a conductivity detector, suppressor and RI-101 detector (Shodex, Germany) (for ethanol detection). The system was maintained at 30 °C using heptafluorobutyric acid (1 mM) as mobile phase with a flow rate at 1 mL/min. Whereas, tetrabutylammonium hydroxide (5 mM) was used as anion suppression reagent. Carbohydrates were detected using a Carbopac PA20 column (Dionex, USA) combined with an electrochemical detector ICS-5000(Dionex, USA). Water (A and B), 100 mM NaOH (C) and 1 M Na acetate were used as solvents using the following gradient: 0 min, 37.5 % B and 25.5 % C; 24 min, 100 % C; 34 min 100 % D; 44 min, 37.5 % B and 25.5 % C. The method for carbohydrates detection was performed according to Schwab et al., (2008).

Volatile compounds from sourdoughs were detected using the method of Gutsche et al. (Gutsche et al., 2012) adapted for doughs. A 75 µm SPME fibre type carboxen/polydimethylsiloxane (Supelco, USA) coupled with a GC–MS 7890A-5975C (Agilent, USA) was used. A qualitative analysis was performed comparing mass spectral data with those of the library.

2.11 Analysis of free amino acids

Free amino acids were determined to observe the AA metabolism of LAB and to monitor peptidase activity. This type of approach was applied by different authors (Cagno et al., 2002; Thiele et al., 2002).

Samples for free amino acids analysis were prepared diluting 500 µL dough with 500 µL distilled water and then 100 µL perchloric acid (30%). Samples were stored overnight at 4 °C to induce protein precipitation. After centrifugation at 15,000 x g for 10 min, the supernatant was used for analysis. Free AA were determined by reversed phase high performance liquid chromatography (RP-HPLC) on an UltiMate 3000 HPLC system (Dionex, Germany) as described by Schurr et al. (2013).

2.12 Analysis of free amino nitrogen

The proteolysis process is normally monitored using the so called ninhydrin test, which determines the concentration of free amino nitrogen (FAN). This approach is often used in sourdough fermentations, as also shown by other authors (Loponen et al., 2009, 2008, 2007; Thiele et al., 2002; Wieser et al., 2007).

The ninhydrin test was performed according to the method of Thiele et al. (2002). Before analysis's beginning, protein precipitation was performed adding 500 µL (7%) of perchloric acid to 500 µL dough and it was stored at 4 °C overnight. Afterwards, the sample was centrifuged at 16,000 x g for 10 min. The measurements were carried out using a FLUOstar Omega at a wavelength of 570 nm (BMG-Labtech, Germany). A distilled water solution was employed for the ninhydrin reaction and used as blank.

2.13 Determination of free thiol groups in buckwheat SDS-soluble fraction

This approach was employed by other authors to monitor the redox potential state of SD and thiol content of SDS-soluble protein fraction (Jänsch et al., 2007; Loponen et al., 2008; Vermeulen et al., 2006).

Free SH groups were measured according to the method of Jänsch et al. (2007) optimized for buckwheat doughs. Dough samples were extracted vortexing for 1 h with a sodium phosphate solution 50 mM (pH 6.9) containing 1 mM EDTA and 1% sodium dodecyl sulfate

(SDS), using an extraction ratio 1:10 (wt/vol). After the extraction, samples were vortexed and 200 µL were used for chemical reaction with Ellman's reagent, 5,5′-Dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich, Germany), while other 200 µL was used for the blank. To obtain the blank of each sample, the normal procedure was performed without Ellman's reagent. The incubation time with Ellman's reagent was 45 min and measurements were performed using a FLUOstar Omega at a wavelength of 412 nm (BMG-Labtech, Germany).

2.14 Protein extraction and Lab-On-Chip capillary electrophoresis

The whole protein fraction of doughs was extracted according to the method of Moroni et al. (2010). This method was adapted for buckwheat and rice dough. Proteins were extracted from each dough for 1 h (extraction ration 1:6 vol/vol) using a sodium phosphate solution 50 mM (pH 6.9) containing 1 mM EDTA and 1% SDS. Afterwards, protein fractions were dialyzed against distilled water, freeze dried and re-dissolved in a 6 M urea phosphate buffer, according to Renzetti et al. (2008a).

Protein extraction from batters was performed adding 1 g BB batter to 9 ml sodium phosphate solution 50 mM (pH 6.9) containing 1 mM EDTA and 1% SDS, while 1 g BR batter was added to 9 ml sodium phosphate solution 50 mM (pH 6.9) containing 1 mM EDTA, 1% SDS and 1 M thiourea. Samples were collected before and after proofing time. After 1 h extraction, samples were centrifuged at 15,000 rpm for 20 min and the supernatant was used for capillary electrophoresis.

A Protein80 Kit (Agilent Technologies, USA) was employed for buckwheat and brown rice dough due to its high sensitivity. Protein fractions were analyzed under reducing conditions (1 M DTT) and non-reducing conditions.

2.15 Fundamental rheology

The rheological properties of batters were tested using a controlled strain and stress rheometer MCR 301 (Antoon Paar, Germany). Batters were prepared without yeast addition and proofed for 40 min. The Linear visco-elastic region was determined by amplitude sweeps between 0.001-100% strain. Whereas, frequency sweeps were performed with a 0.01% strain in the frequency range of 1-50 (Hz) (Moroni et al., 2011). Complex modulus (|G*|) values were fitted using a weak gel model according to the power law equation (Gabriele et al., 2001)

$$G^*(\omega) = A_f \omega^{1/z} \qquad \textbf{Equation 4}$$

where A_f is the network strength and z is the network connectivity.

Temperature sweeps were performed at 0.01% strain with a frequency of 1 Hz in the range 30-90 °C, using a gradient of 0.25 C/s. Temperature was held constant at 90 °C for 2 min (strain 0.01%) and it was cooled down from 90 to 30 °C in 3 min (strain 0.01%).

2.16 Bread making

Two breads were prepared, one using buckwheat SD and buckwheat flour (BB) and the second using buckwheat SD and brown rice flour (BR). Breads were baked without the addition of hydrocolloids to observe the effect of SD on GF breads.

112 g of SD (DY 350), 48 g flour (buckwheat or brown rice), 2 g dry yeast, 1.92 g salt and 0.96 g sugar were added for the bread preparation. Ingredients were mixed for 2 min using a handmixer (TESCO, UK) at maximal speed. 50 g batters were placed into fat-sprayed muffin tins and proofed at 30 °C (85% rh) for 40 min. The baking process was carried out at 230 °C (for top and bottom heat) for 10 min. Steam was injected into the oven as described by Moroni et al. (2011). Loaves were cooled at room temperature for 60 min.

Control bread (Control) was prepared with buckwheat fresh pre-dough, while reducing control bread (C-GSH) was prepared with buckwheat pre-dough containing 3 mM glutathione (GSH). Furthermore, chemically acidified bread was made using chemically acidified doughs (CA), while SD bread was prepared using sourdoughs fermented with *P.*

pentosaceus TMW 2.6 (P) and *E. faecalis* TMW 2.630 (F) for 8 h (P8 and F8) and 24 h (P24 and F24).

2.17 Bread analysis

Baking tests were performed with three loaves of each bread type for each baking trial (independent replicate). Texture profile analysis (TPA) of bread crumb was tested using a texture analyser TA-XT2i (Stable Micro System, UK) equipped with a 25 Kg load cell and 20 mm aluminium cylindrical probe. TPA measurements were performed at 2.0 mm/s using a trigger force of 20 g to compress 50 % of the center of bread slice, which was 20 mm. Values of each independent replicate were calculated averaging values of three loaves of each independent trial, as also described by Moroni et al. (2011).

Bread crumb was analyzed using a C-cell Imaging System (Calibre Control International Ltd., UK). Cell diameter, wall thickness and number of cells/mm^2 were taken into account. Values of each independent replicate were calculated averaging values of three bread slices of each trial.

2.18 Statistical and data analysis

Each trial of this work was performed at least using three independent replicates (n = 3). However, for the microbial screening, outliers were removed before statistical analysis performed. Statistical analysis was carried out using R software (3.0.2, http://www.r-project.org).

2.18.1 Reduction, acidification and oxygen reduction rate

E_{h7}, pH and pO2 variables were mathematically processed to obtain the reduction rate (dE_{h7}/dt, mV/h) the acidification rate (dpH/dt, pH unit/h) and the oxygen reduction rate (dpO2/h, pO2%/h) according to research works (Cachon et al., 2002; Wick et al., 2003). This type of parameters are very useful for the understanding of kinetics during LAB fermentations. Maximum reduction rate V^r_m (mV/h), maximum acidification rate V^a_m (pH unit/h), maximum oxygen reduction rate O^r_m (pO2%/h) and the time at which theses rates occurred (T^r_m (h), T^a_m (h) and T_{or} (h)) were obtained from the calculation.

2.18.2 Pairwise tests

At first, one-way ANOVA was carried out to check independent variable significance and then pairwise t test with Bonferroni or Tukey Honest Significance Difference (HSD) correction was performed to proof statistical significance over the groups (p-value < 0.05).

2.18.3 Multiple regression

Multiple linear regression was computed using the function ‘lm’ (‘stats’ package) and the significance of the model was tested by ANOVA (α = 0.01).

2.18.4 Linear discriminant analysis

Linear discriminant analysis (LDA) is a statistical method used to discriminate specified groups of microbial ecology's data, as also shown by other authors (Colinet and Renault, 2012; Gobbetti et al., 1995; Ramette, 2007). LDA was computed performing at first principal component analysis (PCA) using the function 'dudi.pca' and then the LDA using the 'discrimin' function. LDA was plotted using the functions 's.class' and 's.corcircle'. Multivariate normality and significance were tested by MANOVA ($\alpha = 0.01$) and Monte Carlo ($\alpha = 0.01$) test using the functions 'manova' and 'randtest' (package 'ade4').

2.18.5 Further R functions

Hierarchical cluster analysis was performed calculating first the Euclidean distance of a matrix and then using the function 'hclust' ('stats' package) combined with the method 'ward' for plotting.

The heat map was computed using the function 'heatmap' ('stats' package) or using the package 'ggplot2'.

Box plots were computed using the function 'boxplot' ('graphics' package) combined with the function 'beeswarm' ('beeswarm' package).

Scatter plots for correlations discovering were computed using the function 'pairs' ('graphics' package).

Parallel coordinates were computed using the function 'parcoord' ('MASS' package). This approach is often used for representation of correlations using many variables.

3 Results

3.1 Effect of LAB on the oxidation reduction potential of buckwheat sourdoughs

The goals of this investigation were to determine the effect of LAB growth and metabolism on the ORP, correlated with pH and pO_2 during buckwheat SD fermentations.

The time course of ORP measurements upon fermentation can be divided in three steps: lag phase (0 and 1.5 h), exponential phase (2 and 5 h) and stationary phase (6 and 8 h) (Fig. 5). The first part of the fermentations showed aerobic conditions, while the second part showed anaerobic conditions, in which oxygen reduction occurred (Fig. 5). Sterile control dough displayed a constant concentration of DO (data not shown), indicating no microbial growth. Employed *Lactobacillus* strains showed different reducing activities. Sterile dough exhibited no ORP and pH changes (Fig. 5). Initial ORP differed in each fermentations but it is not statistically significant except for *L. plantarum* (Table 3). *W. cibaria* exhibited the highest reducing activity compared to other strains, reaching a minimal E_{h7} of 9 mV (Fig. 5). Whereas, *P. pentosaceus* displayed the lowest reducing activity with a minimal E_{h7} of 178.3 mV. Each fermentation reached different final E_{h7}, which was statistically significant ($p < 0.05$), except for *L. plantarum* and *L. sakei* (Table 3).

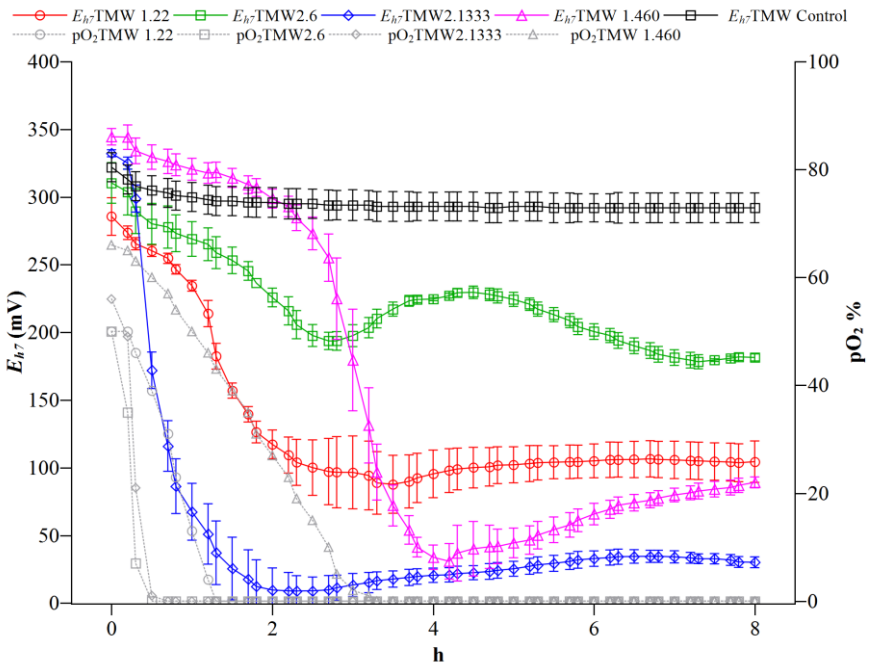

Fig. 5: ORP and pO_2 measurements (n = 3) in buckwheat fermentations with *W. cibaria* TMW 2.1333, *L. plantarum* TMW 1.460, *L. sakei* TMW 1.22, and *P. pentosaceus* TMW 2.6. Max pO_2% standard deviation of each strain was: ± 14 %, ± 9 %, ± 14.4 % and 2.9 %.

A decrease of ORP was observed only after DO consumption in each fermentation (Fig. 5). Moreover, strains with high microbial growth (*W. cibaria* and *L. plantarum*) displayed a better reducing activity compared to the others (Fig. 5 and 6). Maximal acidification activity occurred after the DO consumption and ORP decrease, i.e. between 2 and 4 h in all fermentations. Furthermore, *L. plantarum* was the only strain able to acidify upon aerobic condition (Fig. 5 and Fig. 7). Since the oxygen consumption occurred in each fermentation, the different time course of ORP was correlated to the specific strain activity.

Table 3: initial ORP (E_{h7}s), lowest ORP (E_{h7}m), and final ORP (E_{h7}e) values upon fermentation with *W. cibaria* TMW 2.1333, *L. plantarum* TMW 1.460, *L. sakei* TMW 1.22 and *P. pentosaceus* TMW 2.6. Values within columns with the same letter are not significant ($p < 0.05$). Pairwise comparisons were performed using Bonferroni correction.

Strains	E_{h7}s (mV)	E_{h7}m (mV)	E_{h7}e (mV)
TMW 2.1333	$332.3^{a} \pm 2.6$	$9^{a} \pm 11.3$	$30.2^{a} \pm 4.0$
TMW 1.460	$344.5^{b} \pm 6.4$	$30.9^{a} \pm 13.1$	$89.2^{b} \pm 4.0$
TMW 1.22	$285.6^{c} \pm 13.9$	$87.8^{b} \pm 21.4$	$104.5^{b} \pm 15.3$
TMW 2.6	$310.4^{a,c} \pm 15.0$	$178.3^{c} \pm 5.2$	$181.5^{c} \pm 2.6$

Sucrose, glucose, fructose and maltose were detected in fresh buckwheat dough at the following concentrations: 5.07 ± 0.18, 1.09 ± 0.14, 0.14 ± 0.01 and 0.1 ± 0.01 g/kg flour, respectively. *W. cibaria* and *L. plantarum* used the major part of total carbohydrates, 94.3 and 73 % (growing on sucrose, maltose, glucose and fructose). Whereas, *L. sakei* and *P. pentosaceus* used only 60 and 46.7 % (growing on sucrose, maltose and fructose) of total carbohydrates.

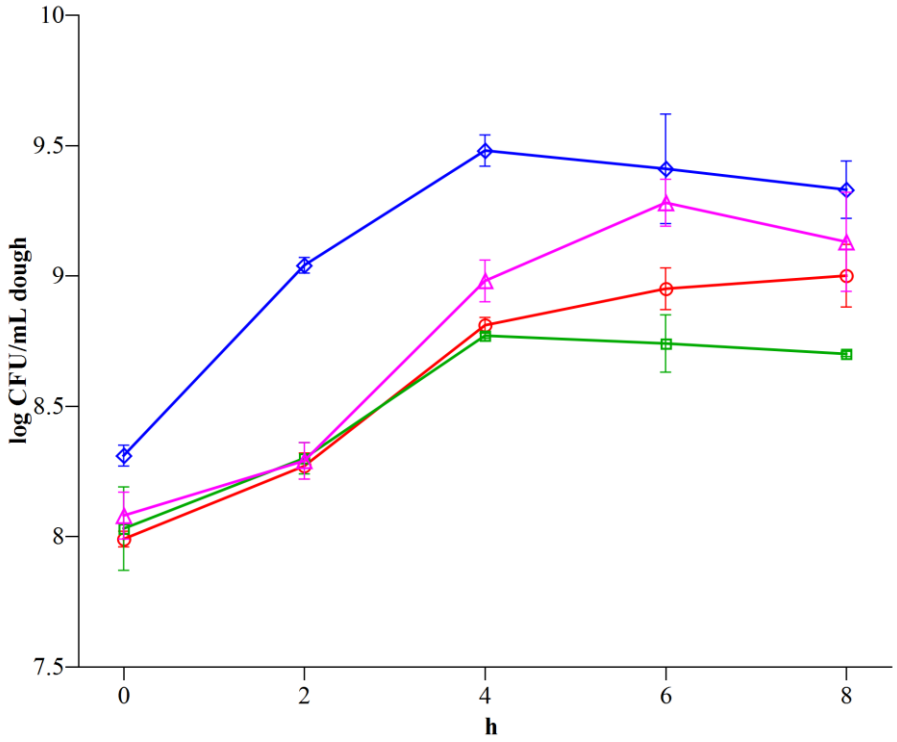

Fig. 6: Microbial counts ($n = 3$) in buckwheat sourdoughs with *W. cibaria* TMW 2.1333, *L. plantarum* TMW 1.460, *L. sakei* TMW 1.22 and *P. pentosaceus* TMW 2.6.

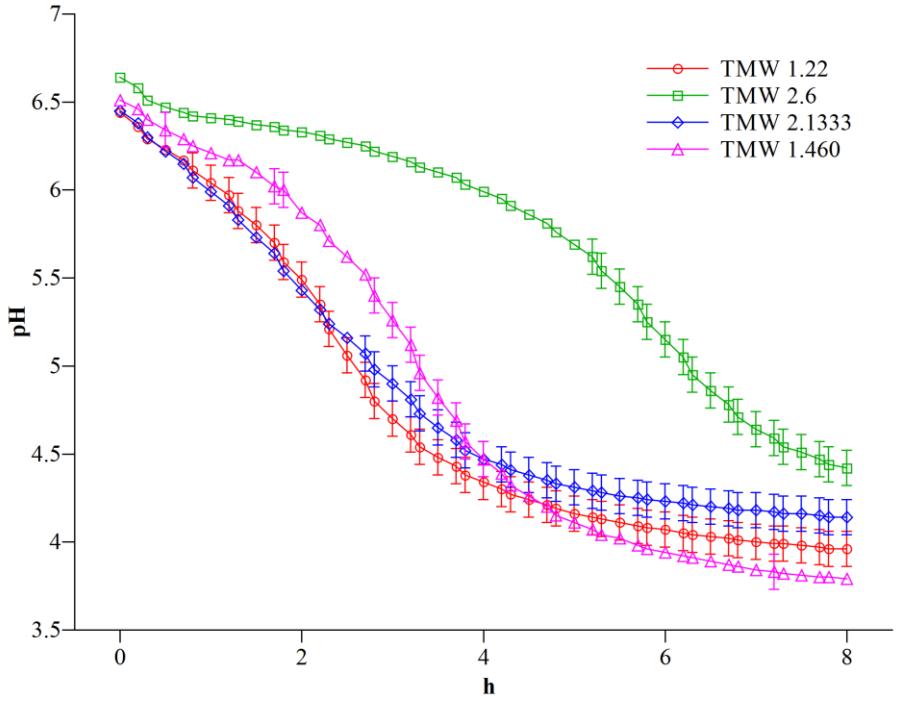

Fig. 7: pH curves (n = 3) of fermentations in buckwheat sourdoughs with *W. cibaria* TMW 2.1333, *L. plantarum* TMW 1.460, *L. sakei* TMW 1.22 and *P. pentosaceus* TMW 2.6.

Fig. 8 displays LAB metabolic activity and reducing activity. Upon the reducing step, a high metabolic activity was occurred and afterwards it decreased. *W. cibaria* and *L. sakei* produced the highest acid content at 2 h after the reducing step and the same event occurred at 4 h in fermentations with *L. plantarum* and *P. pentosaceus* (Fig. 8). Only two type of metabolisms were observed: production of lactic acid, acetic acid, and ethanol by *W. cibaria*, while a production of lactic acid and acetic acid was performed by *L. plantarum*, *L. sakei* and *P. pentosaceus* (Table 4). *W. cibaria* and *L. plantarum* displayed the highest production of organic acids after 8 h (Table 4). Moreover, these strains exhibited the highest reducing activity (Fig. 5 and Table 3) upon fermentations.

Table 4: organic acids and ethanol (n = 3) produced after 8 h in fermentations with *W. cibaria* TMW 2.1333, *L. plantarum* TMW 1.460, *L. sakei* TMW 1.22, and *P. pentosaceus* TMW 2.6. Values within columns with the same upper script letter are not significant ($p < 0.05$).

Strains	Lactic acid (mM)	Acetic acid (mM)	Ethanol (mM)
TMW 2.1333	$92.3^{a} \pm 3.5$	$5.2^{a} \pm 0.5$	$53.6^{a} \pm 1.9$
TMW 1.460	$134.9^{b} \pm 3.3$	$2.3^{b} \pm 0.3$	0^{b}
TMW 1.22	$121.6^{c} \pm 2.7$	$3.4^{b} \pm 0.2$	0^{b}
TMW 2.6	$109.3^{d} \pm 2.6$	$2.7^{b} \pm 0.9$	0^{b}

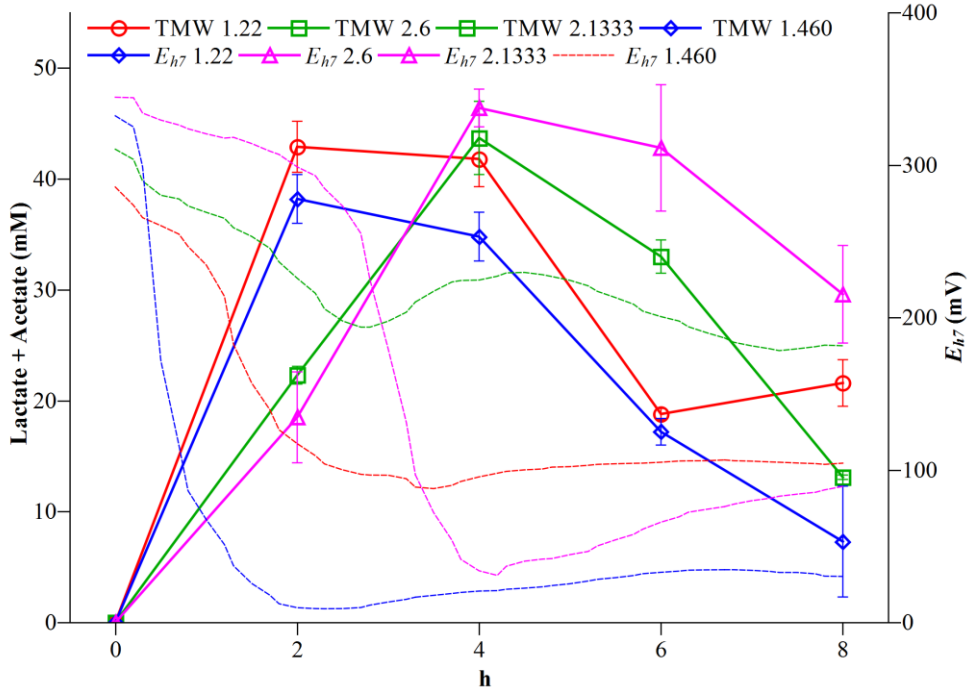

Fig. 8: Production rate of organic acids (n = 3) each 2 h (lactic acid + acetic acid mM) in fermentations with *W. cibaria* TMW 2.1333, *L. plantarum* TMW 1.460, *L. sakei* TMW 1.22, and *P. pentosaceus* TMW 2.6.

W. cibaria displayed a statistical significant difference of V^r_m compared to the other strains ($p < 0.05$) and showed the highest one, as reported in Table 5. The V^r_m occurred after the O^r_m, while the V^a_m occurred after the V^r_m in all fermentations, except for *L. plantarum*, which reached a V^a_m almost at the same time of V^r_m (Table 5 and Fig. 9c).

Table 5: Reduction, acidification and DO consumption rates (n = 3) of fermentations with *W. cibaria* TMW 2.1333, *L. plantarum* TMW 1.460, *L. sakei* TMW 1.22, and *P. pentosaceus* TMW 2.6. Values within columns with the same letter are not significant ($p < 0.05$).

Strains	V^r_m (mV/h)	T^r_m	O^r_m (pO2%/h)	T_{or}	V^a_m (pH/h)	T^a_m
TMW 2.1333	-759.5^a ± 123.6	0.5^a ± 0	-165.5^a ± 54.6	0.3^a ± 0	-0.68^a ± 0.03	2.1^a ± 0.1
TMW 1.460	-323.8^b ± 12.1	3.1^b ± 0.1	-28.3^b ± 3	2.8^b ± 0.1	-1.12^b ± 0.33	2.9^{a,b} ± 0.8
TMW 1.22	-188.6^c ± 6	1.3^c ± 0	-62.1^c ± 13.6	1.1^c ± 0.1	-0.88^b ± 0.03	2.4^b ± 0.1
TMW 2.6	-90.4^d ± 19	0.9^{a,c} ± 1	-164.5^a ± 11.1	0.3^a ± 0	-0.62^a ± 0.03	6^c ± 0

P. pentosaceus was the only strain, which reached the V^a_m later than the other strains (Fig. 9b and Table 5). The V^r_m was not correlated with the V^a_m, thus with the highest metabolic activity. Moreover, even the microbial maximal growth rate was not correlated with the V^r_m (data not shown). However, the reduction activity started with the increase of the acidification in all fermentations (Fig. 9). Upon fermentation, two distinct reduction and acidification steps were detected. The first occurred at one hour after inoculation, while the second was observed between 2 and 6 h (Fig. 9). However, even if the V^r_m and V^a_m did not occur at the same time, a similar trend of reduction and acidification rate was monitored in fermentations with *L. plantarum*, *P. pentosaceus*, and *L. sakei* (Fig. 9b,c and d).

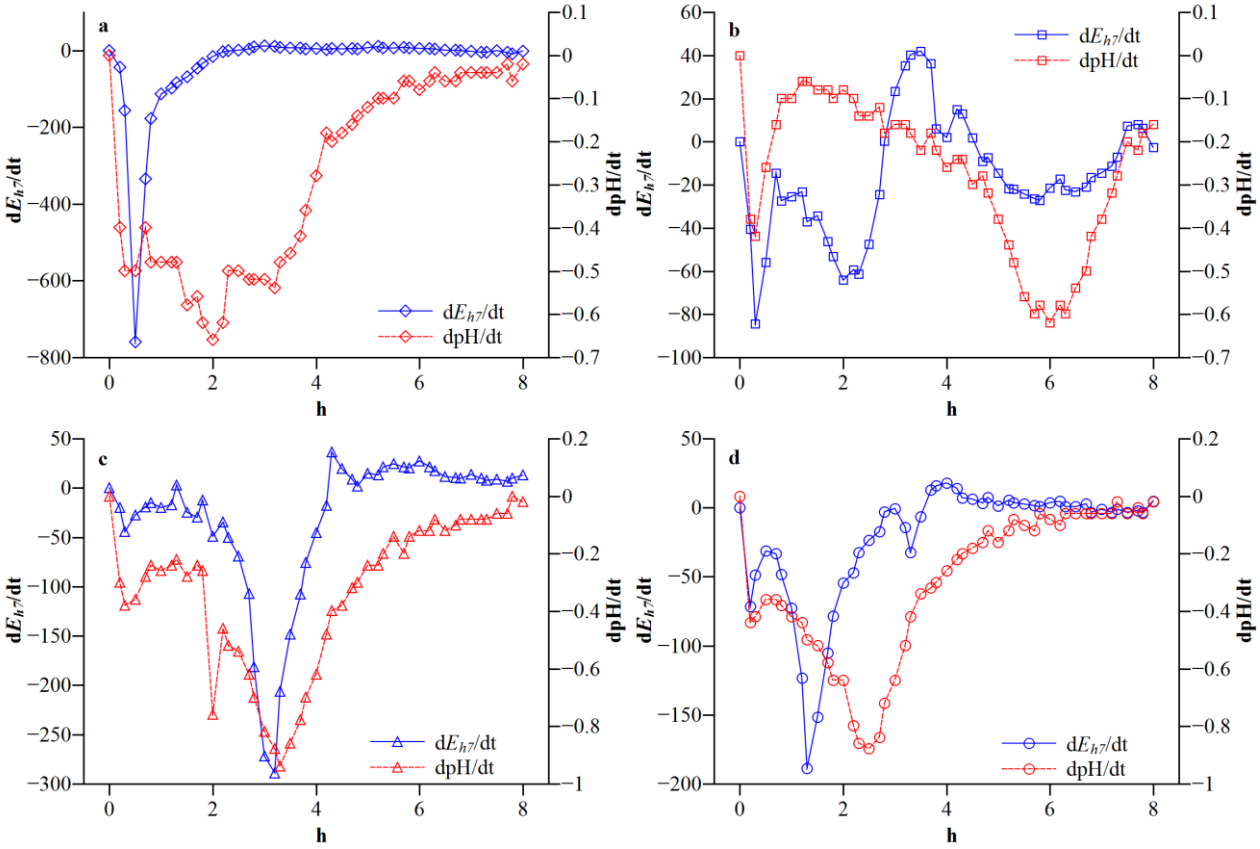

Fig. 9: acidification rate (dpH/dt) and reduction rate (dE_{h7}/dt) (n = 3) of fermentations with *W. cibaria* TMW 2.1333 (a), *L. plantarum* TMW 1.460 (c), *L. sakei* TMW 1.22 (d) and *P. pentosaceus* TMW 2.6 (b).

3.2 Effect of LAB on redox status and proteolysis in buckwheat sourdoughs

Nowadays, the synergic effect between proteolysis and reducing condition in wheat SD is well known. The reducing activity of LAB helps gluten depolymerization, and thus the proteolytic activity (Gänzle et al., 2008). The same behavior could be assumed for buckwheat SD but that should be proven. Indeed, there is a lack of information about the effect of microbial activity on redox status and proteolysis in GF sourdoughs. Probably, the application of SD with different thiol content and proteolysis could have an influence on the finally GF bread characteristics. Consequently, the observation of these phenomena could be helpful to deliver more information about the interaction between LAB and protein breakdown. The goals of this investigation were to observe the effect of reducing and low reducing strains on thiol content and proteolysis in buckwheat sourdoughs. Moreover, fermentations were compared to chemically acidified doughs.

Extracellular proteolytic activity of the employed strains was screened using caseinate agar plates. *E. faecalis* (TMW 2.630) exhibited such activity as also shown by Wieser et al. (2007), thus this strain was used as proteolytic reference. *W. cibaria* (TMW 2.1333) and *P. pentosaceus* (TMW 2.6) showed no proteolytic activity due to the absence of bright ring.

Doughs were inoculated with a cell density of ca. 1 x 10^8 CFU/mL. Maximal microbial growth was determined between 4 and 6 h and afterwards a slight decrease in cell counts was observed in each fermentation (Fig. 10). *W. cibaria* exhibited the highest cell density, 9.13 ± 0.04 log CFU/mL dough. Maximal acidification rate (microbial activity) was achieved between 1 and 3 h upon sourdough fermentations (data not shown). After 8 h a low metabolic activity was observed and therefore a very slow acidification step occurred. Fermentations with *E. faecalis* and *P. pentosaceus* did not produce ethanol, while fermentations with *W. cibaria* and Mix culture exhibited ethanol production (Table 6). Strains with high TTA values (*P. pentosaceus* and Mix culture) showed a higher organic acid production and lower pH values compared to the others (Table 6). *E. faecalis* produced its highest amount of organic acids until 8 h (Table 6) followed by a decreased production rate (data not shown). Cell counts of CA doughs did not exceed 10^4 CFU/mL and yeasts were not detected either in CA or SD.

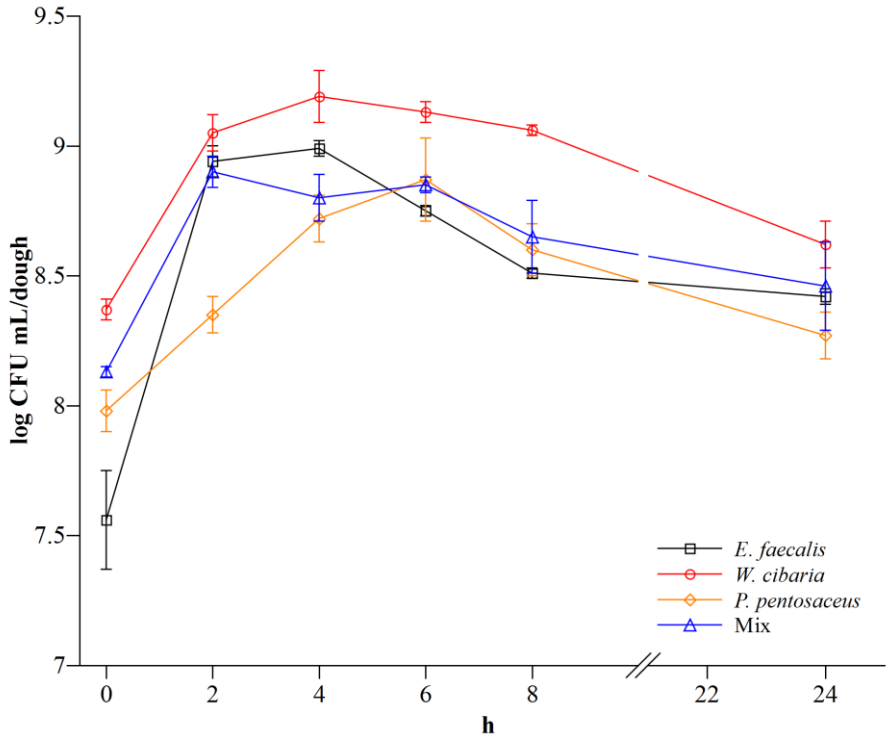

Fig. 10: cell counts (log CFU/mL dough) of buckwheat fermentations (n = 3) with *E. faecalis* (TMW 2.630), *W. cibaria* (TMW 2.1333), *P. pentosaceus* (2.6), and Mix culture (a mix of *P. pentosaceus* and *W. cibaria*).

Table 6: organic acids, ethanol, total titratable acidity (TTA) and pH (n = 3) in buckwheat fermentations with *E. faecalis* (TWM 2.630), *W. cibaria* (TMW 2.1333), *P. pentosaceus* (TMW 2.6), and Mix culture (mix *P. pentosaceus*/*W. cibaria*). Values within columns with the same letter are not significant ($p < 0.05$).

Strains	Lactic acid (mM)		Acetic acid (mM)		Ethanol (mM)	
	8h	**24h**	**8h**	**24h**	**8h**	**24h**
E. faecalis	$52.0^{a} \pm 7.5$	$60.7^{a} \pm 1.8$	$5.2^{a} \pm 0.7$	$7.4^{a} \pm 0.6$	-	-
W. cibaria	$57.8^{b,a} \pm 7.6$	$77.8^{b} \pm 5.8$	$7.6^{b} \pm 0.5$	$9.8^{b,a} \pm 1.4$	$22.5^{a} \pm 6.1$	$88.0^{a} \pm 4.4$
P. pentosaceus	$70.5^{b} \pm 4.5$	$112.8^{c} \pm 3.7$	$6.5c^{a} \pm 0.5$	$11.8^{b,c} \pm 0.6$	-	-
Mix	$77.0^{b} \pm 2.0$	$108.7^{c} \pm 4.0$	$7.8c^{b} \pm 0.5$	$13.0^{c} \pm 0.9$	$11.8^{b} \pm 3.6$	$17.0^{b} \pm 2.6$

	TTA (mL)		pH	
	8h	**24h**	**8h**	**24h**
E. faecalis	$2.20^{a} \pm 0.05$	$2.43^{a} \pm 0.03$	$4.31^{a} \pm 0.03$	$4.22^{a} \pm 0.03$
W. cibaria	$2.35^{b,a} \pm 0.13$	$3.23^{b} \pm 0.06$	$4.2^{a} \pm 0.03$	$3.98^{b} \pm 0.02$
P. pentosaceus	$2.62^{b} \pm 0.18$	$3.78^{c} \pm 0.19$	$3.96^{b} \pm 0.03$	$3.71^{c} \pm 0.05$
Mix	$2.95^{c} \pm 0.05$	$4.20^{d} \pm 0.10$	$4.01^{b} \pm 0.09$	$3.77^{c} \pm 0.1$

ORP and free thiols were measured during the whole fermentation time. The reducing activity of employed strains can be summarized as follows: *E. faecalis* > *W. cibaria* > Mix > *P. pentosaceus* > CA. After fermentation, the initial E_h decrease occurred during the first hours (0-2 h) except for the CA (Fig. 11e). Moreover, after the reduction step a slightly ORP increase (4-8 h) was observed except in fermentations with *E. faecalis* (Fig. 11a). The same ORP trend occurred between 8 and 24 h in all fermentations, including CA, (data not shown). *E. faecalis*, *W. cibaria*, *P. pentosaceus*, Mix culture, CA+GSH and CA doughs reached at

24 h a final E_h of: -20 ± 13, 202 ± 11, 321 ± 7, 332 ± 9, 350 ± 16 and 445 ± 22 mV, respectively. Besides, oxygen intrusion was not detected in sourdough fermentations and during microbial growth the oxygen content was reduced. However, in CA and CA+GSH doughs, the oxygen was not reduced and it increased over the time. Free SH groups increased during the reduction step and decreased when the E_h become oxidative in SD fermentations with *E. faecalis*, *W. cibaria*, and *P. pentosaceus* (Fig. 11a,b,c). Whereas, fermentations with the Mix culture and CA doughs displayed a decreasing trend of thiol content (Fig. 11d,e). The most reducing strains, *E. faecalis* and *W. cibaria*, showed higher thiol contents at 8 h (2.38 ± 0.17 and 2.14 ± 0.3 mmol/Kg flour), instead *P. pentosaceus*, less reducing, showed the lowest mean value (0.91 ± 0.26 mmol/Kg flour). However all fermentations, including CA doughs, exhibited a similar decrease of free thiol groups after 24 h of approximately 1 mmol/Kg.

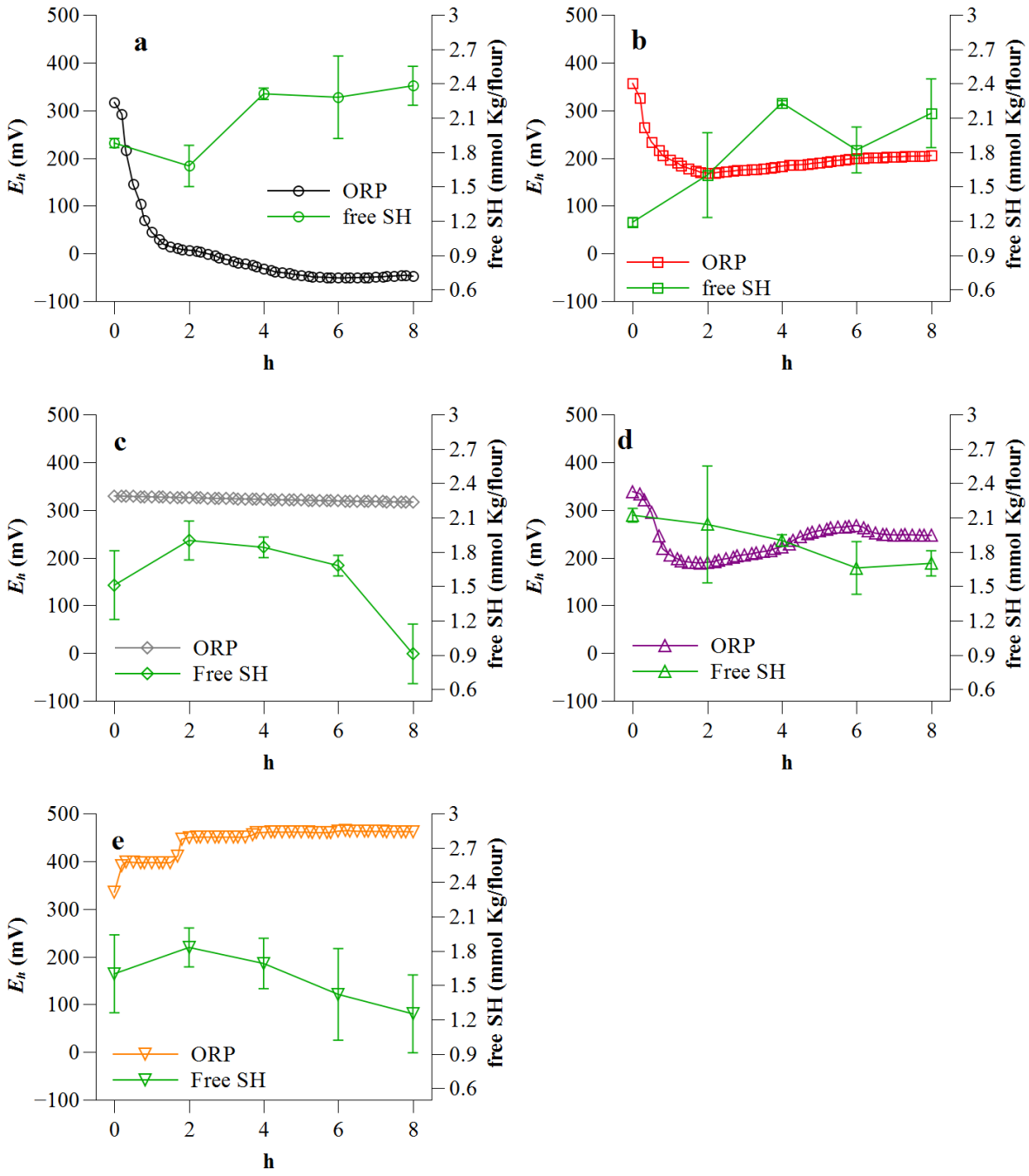

Fig. 11: ORP time course and free SH content in buckwheat fermentations (n = 3) with *E. faecalis* (TMW 2.630) (**a**), *W. cibaria* (TMW 2.1333) (**b**), *P. pentosaceus* (2.6) (**c**), Mix culture (a mix of *P. pentosaceus* and *W. cibaria*) (**d**) and CA doughs (**e**).

To understand which effect pH and endogenous proteases have on proteolysis, the trials were carried out without microbial inoculation in buckwheat doughs. The influence of pH on proteolysis was detected between pH 6.9 and 2.8 (Fig. 12). As the pH of the dough was decreased from 6.9 down to 3.3, an increase of proteolysis was displayed in the first trial (Fig. 12) but this was not statistical significant ($p < 0.05$) against the start point (pH 6.9). At pH 2.8 a clear activity reduction was observed. The same trend was monitored in the second trial but in this case a non-significant ($p < 0.05$) decrease of activity has been shown at pH 3.9 (Fig. 12).

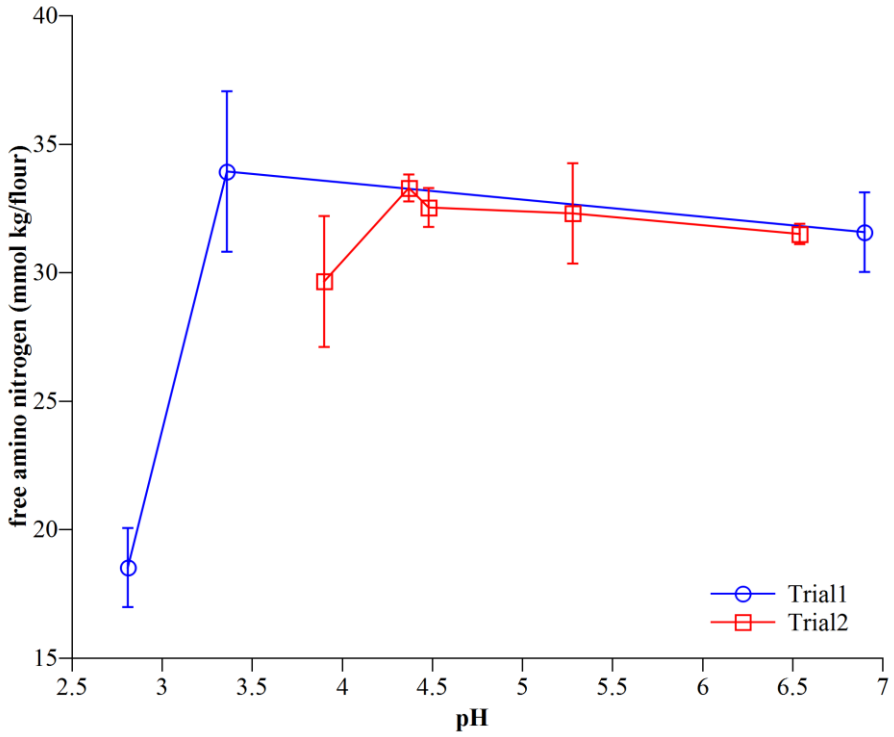

Fig. 12: Influence of pH on proteolysis in buckwheat doughs (non-inoculated doughs) after 24 h at 30 °C. Due to the non-uniformity of buckwheat flour, two trial ($n = 3$) with different batches of buckwheat were performed. Soughs of DY 500.

The addition of protease inhibitors showed a reduction of FAN compared to the control (Fig. 13), indicating the presence of metalloproteases, aspartic, cysteine, and serine proteases in buckwheat flour. Except the E-64, other inhibitors displayed a significant reduction ($p < 0.05$) of the proteolysis (Fig. 13). The relative contribution of each endogenous protease could be weighted to a trend as follows: metalloproteinases > serine proteases > aspartic proteases > cysteine proteases. However, comparing the inhibitory effect using a pairwise *t* test, there is no statistical significance ($p < 0.05$) over the inhibitors.

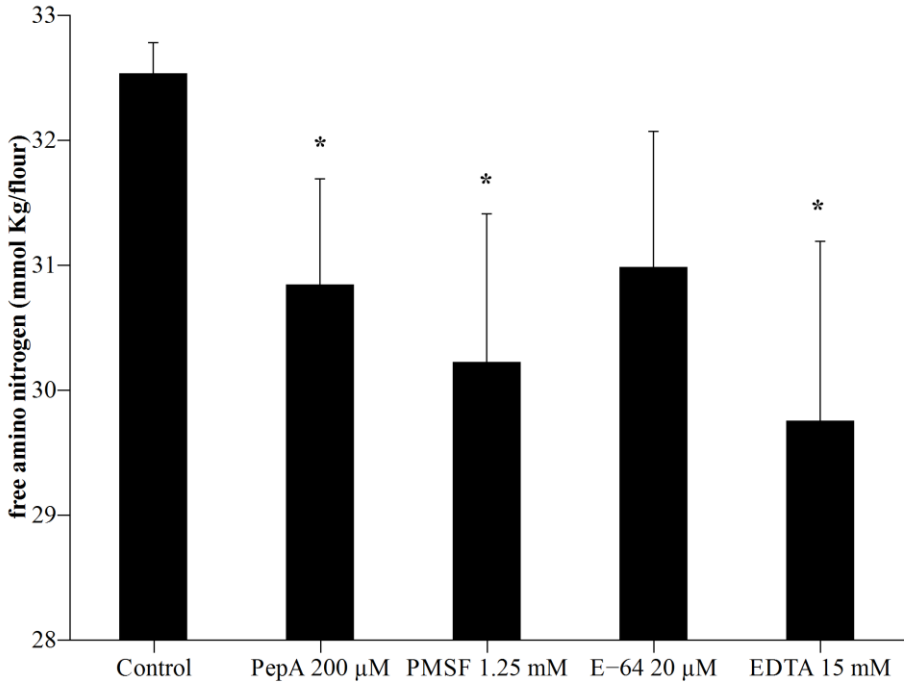

Fig. 13: Effect of inhibitors on the endogenous proteases of buckwheat dough (DY500) after 24 h at 30 °C and pH 6.3. PepA: pepstatin A (inhibitor of aspartic proteases), PMSF: phenylmethylsulfonyl fluoride (inhibitor of serine proteases), E-64 (inhibitor of cysteine proteases) and EDTA: ethylenediaminetetraacetic acid (inhibitor of metalloproteinases). *: significance compared to the Control dough ($p < 0.05$).

The proteolysis was measured using a ninhydrin test and it is commonly used to monitor the extent of the overall proteolysis upon sourdough fermentations (Loponen et al., 2007; Thiele et al., 2002). Proteolysis increase was observed in all fermentations, as shown in Fig. 14. Proteolytic strain *E. faecalis* displayed the highest FAN content at 8 h, while the other strains showed a lower content until 8 h (Fig. 14). Moreover, high FAN content was observed during the first hours (2-6 h) in CA doughs (Fig. 14). A slow increase over time was only monitored in CA+GSH doughs containing 5 mM GSH (Fig. 14). After 24 h, doughs with *P. pentosaceus*, *E. faecalis*, and *W. cibaria* showed the highest content of FAN (45.89 ± 1.32, 42.39 ± 1.31, 40.05 ± 1.06 mmol/Kg flour). Whereas, the CA+GSH doughs exhibited the lowest one instead (17.84 ± 3.86 mmol/Kg flour), i.e., the addition of a reducing compound,

such as glutathione, have an inhibition effect on proteolysis in buckwheat sourdoughs. Multiple regression was performed to understand which environmental variable has a significant effect on proteolysis (expressed as FAN) after 24 h. Values of fermentations with *E. faecalis* were excluded from regression due to the intrinsic proteolytic activity. The linear model explained ca. 70% of variance (R^2= 0.7069). An ANOVA of the model displayed the significant influence of pH (α = 0.01) on proteolysis. Thus, low pH improved the proteolysis over fermentation (between pH 6-3.6). FAA were measured after 24 h in buckwheat sourdoughs. Fermentations with *E. faecalis* exhibited the highest FAA total content, while CA + GSH exhibited a low concentration (Table 7). Moreover, fermentations with *W. cibaria*, *P. pentosaceus* and Mix culture displayed a lower total concentration of total FAA than CA doughs (Table 7).

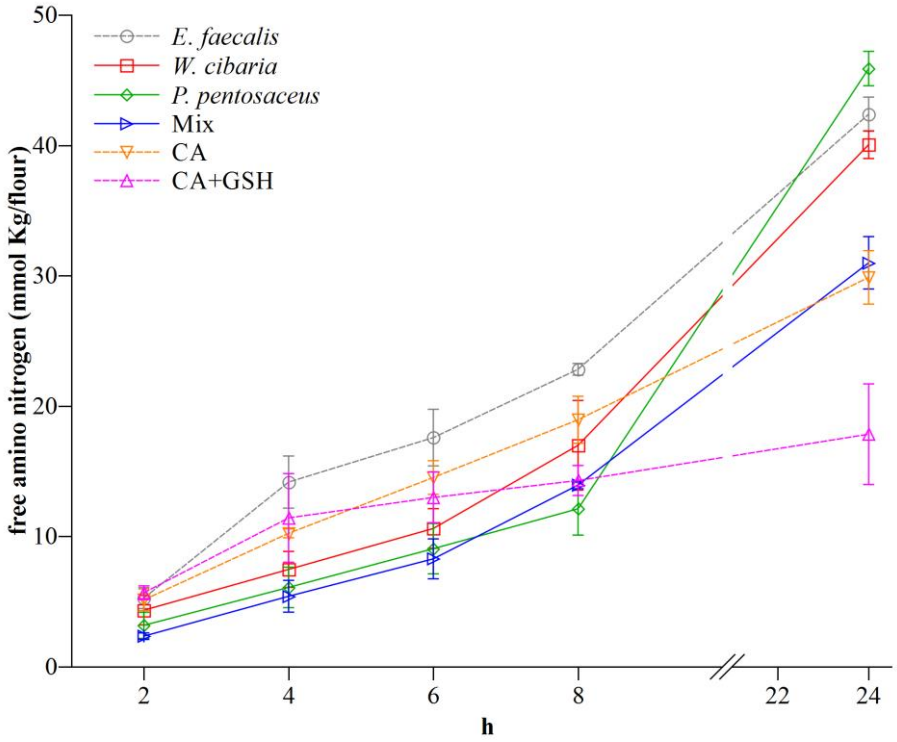

Fig. 14: FAN content during buckwheat fermentations (*n* = 3) with *E. faecalis* (TMW 2.630), *W. cibaria* (TMW 2.1333), *P. pentosaceus* (TMW 2.6), and Mix culture. To remove the difference of initial FAN content in each dough, the first measurement was subtracted from each further value over time.

Table 7: FAA content after 24 h buckwheat fermentation (n = 3) (expressed in mmol/L dough) with *E. faecalis* (TMW 2.630), *W. cibaria* (TMW 2.1333), *P. pentosaceus* (TMW 2.6), and Mix culture (mix of *W. cibaria* and *P. pentosaceus*). Values within columns with the same letter are not significant ($p < 0.05$).

			Fermentations			
Amino acids	**CA**	**CA + GSH**	***E. faecalis***	***W. cibaria***	***P. pentosaceus***	**Mix**
Asp	0.33±0.24	0.75±0.31	1.22±0.22	1.10±0.11	0.48±0.33	1.04±0.06
Glu	0.93±0.30	0.84±0.30	0.68±0.77	0.13±0.03	0.68±0.00	0.20±0.04
Asn	0.92±0.01	0.77±0.11	1.36±0.04	0.72±0.02	0.40±0.01	0.65±0.04
Ser	0.47±0.00	0.21±0.03	0.81±0.03	0.60±0.06	0.21±0.06	0.24±0.01
Gln	0.14±0.04	0.03±0.01	0.12±0.00	0.06±0.01	0.01±0.01	0.02±0.00
His	0.14±0.02	0.44±0.09	0.21±0.00	0.13±0.01	0.09±0.02	0.10±0.00
Gly	0.55±0.03	0.39±0.04	0.64±0.05	0.40±0.03	0.36±0.09	0.40±0.03
Thr	0.28±0.00	0.09±0.01	0.66±0.04	0.35±0.04	0.30±0.09	0.28±0.01
Arg	1.22±0.00	0.83±0.07	0.15±0.02	0.09±0.14	0.03±0.01	0.02±0.00
Ala	1.27±0.04	0.61±0.05	2.47±0.15	0.61±0.52	0.61±0.02	0.78±0.04
Tyr	0.45±0.01	0.28±0.01	0.03±0.01	0.52±0.04	0.39±0.10	0.44±0.02
Cys2	0.58±0.02	0.37±0.02	0.59±0.04	0.38±0.30	0.36±0.08	0.44±0.02
Val	0.69±0.05	0.38±0.07	1.15±0.08	0.82±0.03	0.76±0.01	0.87±0.04
Met	0.38±0.02	0.07±0.03	0.34±0.07	0.41±0.23	0.08±0.02	0.10±0.00
Phe	0.23±0.03	0.17±0.00	0.32±0.03	0.46±0.57	0.20±0.04	0.24±0.01
Trp	0.97±0.02	0.44±0.06	1.11±0.02	0.90±0.30	0.71±0.03	0.95±0.05
Ile	0.49±0.02	0.22±0.03	0.78±0.06	0.88±0.50	0.45±0.01	0.59±0.03
Orn	0.00±0.00	0.00±0.00	1.42±0.12	1.18±0.31	0.59±0.02	0.89±0.05
Leu	2.07±0.61	0.74±0.10	2.21±0.06	1.58±0.40	1.40±0.04	1.91±0.09
Lys	1.09±0.17	0.35±0.05	1.06±0.10	0.61±0.23	0.30±0.09	0.36±0.02
Total	**13.2^{a}±1.0**	**8.0^{b,e}±0.6**	**17.3^{c}±0.6**	**11.9^{d,a}±1.12**	**8.4^{e,f}±0.9**	**10.5^{f,d}±0.5**

The whole protein fraction (SDS-soluble) of buckwheat sour and chemically acidified doughs was analyzed using a Lab-On-Chip technology. Electropherograms of buckwheat fresh doughs displayed bands at 8, 14, 16, 20, 23 and 37 kDa as well as between 50 and 60 kDa under non-reducing conditions (Fig. 15a). On the other hand, bands have been detected at 5, 7, 16 and 21 kDa as well as between 31 and 52 kDa under reducing condition (Fig. 15b). As expected, the band intensity of protein fractions at 24 h is lower than fractions at 8 h (Fig. 15). Moreover, fractions analyzed under reducing conditions displayed another band pattern, showing more low molecular bands than non-reduced fractions (Fig. 15b,d). Proteolytic activity resulted in an modified band patterns compared to the fraction of fresh dough under non-reducing conditions (Fig. 15a,c). This difference was not observed under reducing conditions. Between fermentations and CA doughs, identical patterns were monitored both under reducing and non-reducing conditions (Fig. 15). However, differences

were exhibited in the intensity of the bands over time, especially under reducing conditions (Fig. 15c,d). In this case a thick band occurred at 21 kDa in all fractions.

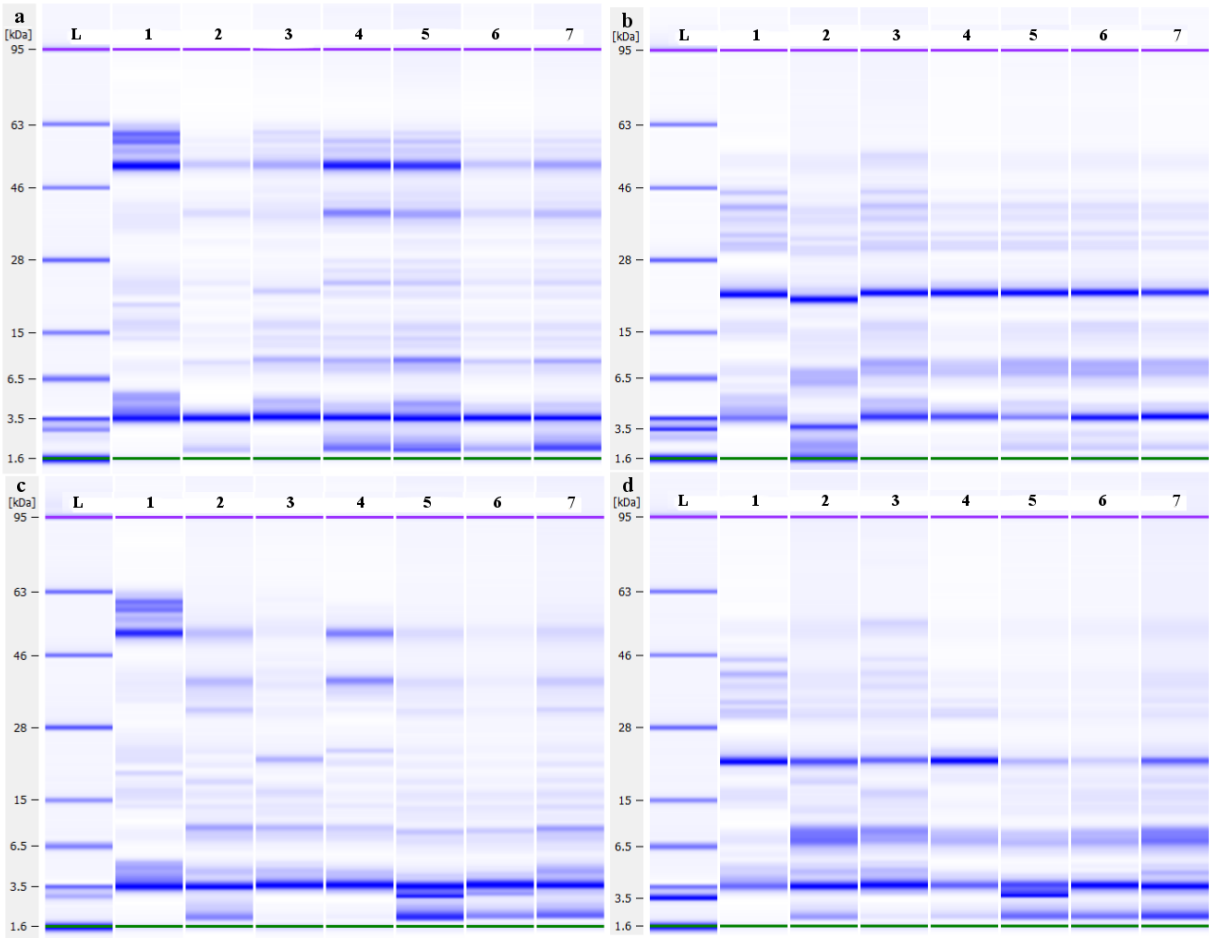

Fig. 15: electrophoretic pattern of non-reduced (**a,c**) and reduced (**b,d**) entire protein fraction of buckwheat doughs at 8 h (**a,b**) and 24 h(**c,d**). The meaning of the legend is the following: (**L**) molecular weight marker; (**1**) buckwheat fresh dough fraction; (**2**) CA; (**3**) CA + GSH; (**4**) *E. faecalis*;(**5**) *W.cibaria*; (**6**) *P. pentosaceus*; (**7**) Mix culture. Fractions were extracted from a dough mix of three independent replicates.

3.3 Effect of controlled extracellular ORP on microbial metabolism, thiol content and proteolysis in buckwheat SD

The aim of this investigation was to observe the effects of controlled extracellular ORP in buckwheat SD fermentations, detecting metabolite production, changes in free thiol content, release of FAA and volatile compounds. For this scope two LAB strains (*Weissella cibaria* TMW 2.1333 and *Pediococcus pentosaceus* TMW 2.6) and a co-culture (composed from these two strains) were employed for fermentations.

The influence of controlled extracellular ORP on LAB activity was determined in buckwheat SD fermentations. The ORP was held constant at ca. 300 and -350 mV in each fermentation, except for *P. pentosaceus* (Fig. 16b) where the ORP increased after 3 h of fermentations. Control fermentations with Mix culture exhibited the highest reducing activity, while fermentations with *P. pentosaceus* showed the lowest one (Fig. 16b,c). Cell density was low in fermentations with mix culture using N_2/H_2 (Fig. 16f). No differences were detected between oxidizing and control fermentations, except for *P. pentosaceus* displaying a high growth yield under oxidizing and reducing conditions (Fig. 16e). The fastest acidification was observed in oxidizing fermentations with *W. cibaria* and mix culture (Fig. 16d,f). Instead, strongly reducing conditions caused a slightly slower acidification in all fermentations (Fig. 16d,e,f). After 8h all fermentations reached similar pH (Fig. 16d,e,f). Normal fermentations with *P. pentosaceus* exhibited the highest content of lactic acid, while normal fermentations with *W. cibaria* showed the lowest one (Table 8). Oxidizing conditions influenced the lactate/acetate ratio of *W. cibaria* and mix culture, because more acetate was produced compared to the control fermentations (Table 8). Reducing conditions did not display an influence on this ratio, and no influence on LAB metabolism was detected (Table 8).

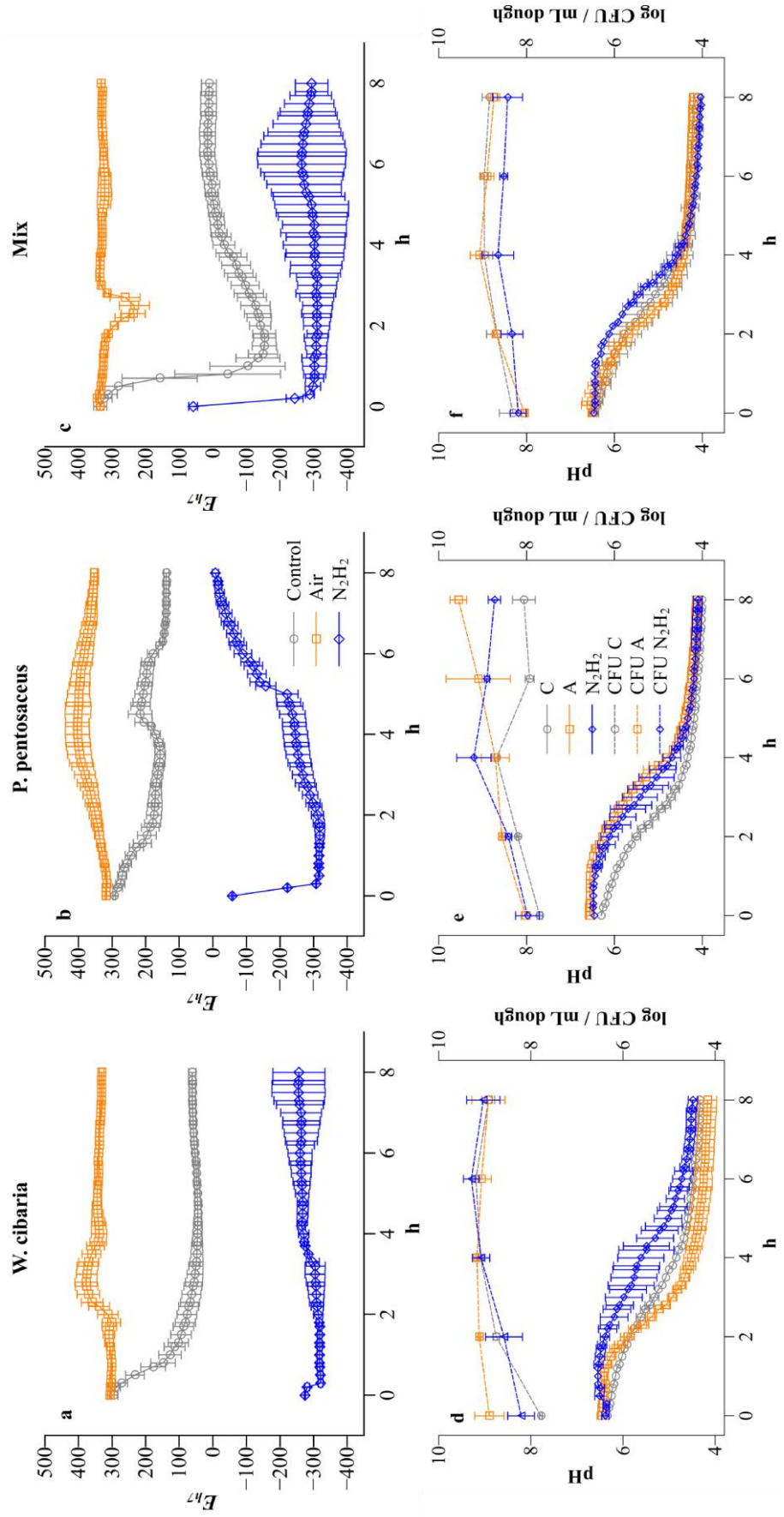

Fig. 16: Colony counts, ORP and pH in fermentations (n = 3) with *W. cibaria* (TMW 2.1333) (**a**,**d**), *P. pentosaceus* (TMW 2.6) (**b**,**e**) and Mix culture (**c**,**f**) under normal (gray lines), oxidizing (orange lines) and reducing conditions (blu lines). Legend specifications: control fermentations (C), oxidizing fermentations (A).

Table 8: content of organic acids, ethanol, and lactic-acetic acid ratio after 8 h in buckwheat fermentations (n = 3) with *W. cibaria* (TMW 2.1333), *P. pentosaceus* (TMW 2.6) and mix culture under control, oxidizing and reducing conditions. Values within columns with the same upper script letter are not significant ($p < 0.05$).

Fermentations	Lactic acid (mM)	Acetic acid (mM)	Ethanol (mM)	lactic/acetic acid
W. cibaria	61.65^{a}±3.22	7.86^{a}±0.99	45.30^{a}±7.78	7.9^{a}±1.3
W. cibaria air	38.33^{b}±3.67	77.20^{b}±4.97	0.00^{b}±0.00	0.5^{b}±0.1
W. cibaria N_2/H_2	52.84^{a}±6.04	10.09^{a}±1.55	51.18^{a}±9.70	5.3^{c}±0.3
P. pentosaceus	74.97^{a}±1.28	6.14^{a}±0.52	0.00^{a}±0.00	12.3^{a}±1.2
P. pentosaceus air	61.46^{b}±3.06	3.65^{b}±0.15	0.00^{a}±0.00	16.8^{b}±1.2
P. pentosaceus N_2/H_2	69.98^{a}±7.10	2.60^{c}±0.32	0.00^{a}±0.00	27.0^{c}±0.6
Mix	67.22^{a}±1.25	4.21^{a}±0.09	25.42^{a}±3.94	16.0^{a}±0.6
Mix air	52.31^{b}±5.79	44.34^{b}±2.32	0.00^{b}±0.00	1.2^{b}±0.2
Mix N_2/H_2	73.21^{a}±3.43	3.49^{a}±0.52	0.00^{b}±0.00	19.8^{a}±3.9

Inositol, glucose, fructose, sucrose, and maltose have been detected in fresh buckwheat doughs (before inoculation). Under normal conditions (without gas sparging), all strains grown mainly on: glucose, fructose, sucrose and maltose (Fig. 17). In all fermentations, a production increase of inositol was observed. During oxidizing conditions a high quantity of glucose was released in sourdoughs, especially by *P. pentosaceus* (Fig. 17b). Moreover, fructose was not consumed under oxidizing conditions in fermentations with *W. cibaria* and *P. pentosaceus* (Fig. 17a,b). Under reducing ORP, a glucose consumption by *W. cibaria* and Mix culture was observed (Fig. 17a,c).

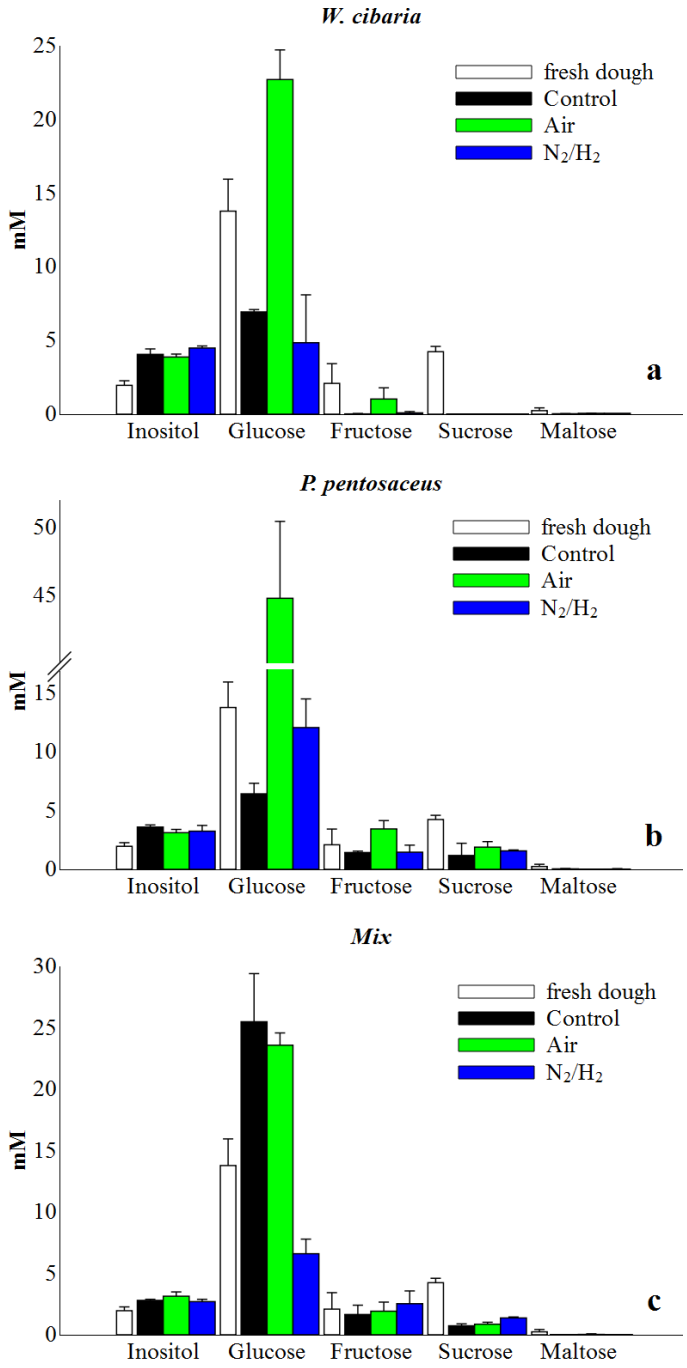

Fig. 17: Carbohydrate's content (n = 3) in buckwheat fresh doughs (time 0) and sourdoughs inoculated (after 8 h) with *W. cibaria* (TMW 2.1333) (**a**), *P. pentosaceus* (TMW 2.6) (**b**) and mix culture (**c**) under control, oxidizing and reducing conditions.

Free thiols of buckwheat SDS-soluble fraction after 8 h fermentation were detected. Comparing all fermentations with the control (without gassing), low values of free thiols occurred under high E_{h7} values (Fig. 18). Fermentations with Mix culture showed the highest free thiol content, while fermentations with *P. pentosaceus* displayed the lowest one (Fig. 18). Under oxidizing conditions, free SH decreased in all trials, especially for Mix culture (Fig. 18). Reducing conditions displayed different results instead, e.g. compared to normal fermentations (without gassing), fermentations with *W. cibaria* did not show significant differences, while a thiol decrease were observed by Mix culture (Fig. 18). Moreover, fermentations with *P. pentosaceus* under reducing conditions displayed a clear thiols increase compared to the control (Fig. 18).

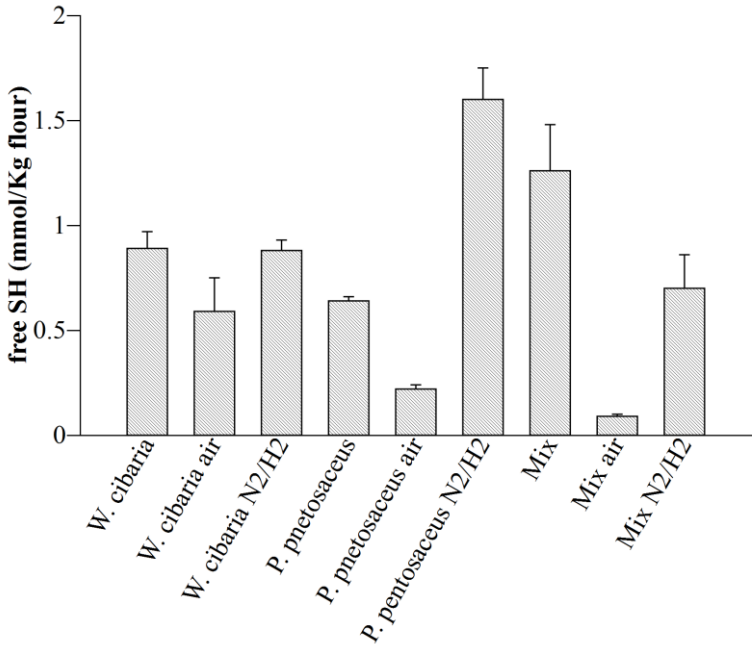

Fig. 18: content of free thiols (n = 3) of buckwheat SDS-soluble fraction after 8 h fermentation with *W. cibaria* (TMW 2.1333), *P. pentosaceus* (TMW 2.6) and mix culture.

Proteolysis was determined measuring the FAN content at the end of fermentations. Control fermentations with Mix culture exhibited the highest FAN content compared to the other control fermentations, with *W. cibaria* and *P. pentosaceus* (Fig. 19). Under oxidizing conditions, a proteolysis decrease was detected in fermentations with *W. cibaria* and Mix culture (Fig. 19). Instead, a significantly ($p < 0.05$) increase of proteolysis occurred only in fermentations with *W. cibaria* under reducing conditions, while for fermentations with Mix culture a decreasing trend was observed (Fig. 19).

FAA content of SD after 8 h was determined (Table 9). A decreasing trend of total FAA has been detected under oxidizing conditions in all fermentations, while fermentations with *P. pentosaceus* showed a significant ($p < 0.05$) decrease (Table 9). Whereas, a significant ($p < 0.05$) increase was recognized in fermentations with *W. cibaria* under reducing conditions (Table 9).

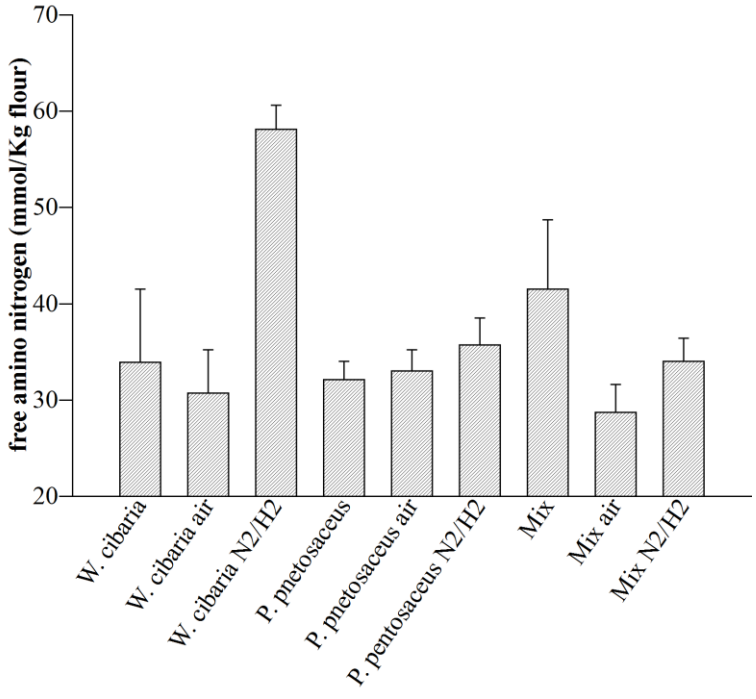

Fig. 19: FAN content after 8 h in buckwheat fermentations ($n = 3$) with *W. cibaria* (TMW 2.1333), *P. pentosaceus* (TMW 2.6) and mix culture.

Mix culture and *P. pentosaceus* displayed similar FAA patterns between control and reducing conditions, while their FAA patterns were completely different under oxidizing conditions compared to other fermentations. *W. cibaria* exhibited similar FAA patterns between control and aerobic conditions. Arginine was not consumed under high oxidizing conditions, except for mix culture. Low ornithine concentrations were produced under oxidizing conditions, especially by *W. cibaria* (Table 9). Moreover, consumption of phenylalanine was detected in each fermentation under aerobic conditions (Table 9).

Table 9: content of FAA and GABA after 8 h buckwheat fermentations with *W. cibaria* TMW 2.1333 (W), *P. pentosaceus* TMW 2.6 (P), and Mix culture (M). Values within last row with the same letter are not statistically significant ($p < 0.05$).

AA	W	W air	W N_2/H_2	P	P air	P N_2/H_2	M	M air	M N_2/H_2
Asp	0.26±0.11	0.20±0.06	0.70±0.21	0.32±0.05	0.27±0.02	0.42±0.04	0.45±0.08	0.20±0.04	0.41±0.06
Glu	0.84±0.28	0.68±0.15	1.88±0.57	0.99±0.15	0.85±0.05	1.07±0.14	1.20±0.26	0.61±0.12	1.26±0.20
Asn	0.33±0.10	0.07±0.02	1.02±0.27	0.16±0.01	0.14±0.00	0.32±0.06	0.34±0.08	0.07±0.01	0.31±0.04
Ser	0.24±0.07	0.16±0.03	0.67±0.20	0.10±0.03	0.07±0.01	0.13±0.01	0.14±0.03	0.06±0.01	0.15±0.04
Gln	0.04±0.01	0.07±0.01	0.40±0.11	0.08±0.04	0.05±0.01	0.04±0.05	0.07±0.02	0.03±0.01	0.08±0.01
His	0.06±0.02	0.04±0.01	0.13±0.04	0.07±0.02	0.07±0.00	0.07±0.00	0.06±0.01	0.03±0.01	0.09±0.02
Gly	0.29±0.05	0.20±0.10	0.53±0.14	0.38±0.07	0.37±0.01	0.40±0.02	0.40±0.05	0.25±0.03	0.45±0.07
Thr	0.16±0.05	0.11±0.08	0.45±0.14	0.17±0.04	0.08±0.02	0.19±0.02	0.14±0.02	0.06±0.01	0.16±0.03
Arg	0.00±0.00	0.18±0.11	0.01±0.00	0.09±0.07	0.25±0.05	0.14±0.14	0.07±0.04	0.01±0.00	0.24±0.18
Ala	0.28±0.07	0.25±0.05	1.08±0.30	0.43±0.08	0.36±0.01	0.50±0.05	0.34±0.07	0.20±0.06	0.35±0.11
GABA	0.28±0.06	0.19±0.10	0.39±0.13	0.31±0.08	0.30±0.01	0.32±0.02	0.35±0.04	0.19±0.04	0.46±0.07
Tyr	0.16±0.03	0.06±0.02	0.48±0.15	0.18±0.03	0.13±0.01	0.20±0.04	0.19±0.05	0.07±0.02	0.18±0.03
Cys2	0.29±0.14	0.26±0.07	0.64±0.21	0.44±0.07	0.46±0.06	0.51±0.06	0.53±0.12	0.27±0.07	0.57±0.11
Val	0.27±0.09	0.15±0.04	0.90±0.27	0.42±0.08	0.31±0.07	0.36±0.04	0.44±0.10	0.15±0.04	0.34±0.06
Met	0.08±0.04	0.04±0.01	0.27±0.08	0.02±0.02	0.04±0.03	0.12±0.06	0.04±0.01	0.03±0.01	0.14±0.03
Phe	0.20±0.17	0.11±0.02	0.17±0.02	0.14±0.03	0.11±0.04	0.14±0.02	0.13±0.02	0.06±0.03	0.15±0.04
Trp	0.17±0.13	0.18±0.04	0.86±0.21	0.40±0.07	0.26±0.04	0.43±0.08	0.41±0.09	0.16±0.02	0.36±0.06
Ile	0.18±0.05	0.10±0.02	0.61±0.15	0.20±0.03	0.15±0.02	0.23±0.04	0.22±0.05	0.08±0.02	0.20±0.03
Orn	0.62±0.16	0.25±0.06	1.10±0.35	0.65±0.11	0.44±0.09	0.59±0.13	0.70±0.13	0.46±0.09	0.59±0.34
Leu	0.55±0.14	0.34±0.07	1.73±0.43	0.76±0.13	0.53±0.05	0.84±0.16	0.81±0.18	0.35±0.08	0.72±0.10
Lys	0.23±0.04	0.11±0.02	0.85±0.19	0.13±0.02	0.13±0.01	0.21±0.03	0.23±0.05	0.07±0.01	0.24±0.05
Total*	**4.96^{a}±1.35**	**3.28^{a}±0.76**	**13.87^{b}±3.82**	**5.70^{a}±1.01**	**4.62^{a}±0.31**	**6.39^{a}±0.75**	**6.39^{a}±1.27**	**2.98^{b}±0.62**	**6.41^{a}±1.06**

*: concentration of total free amino acids without GABA.

During control and reducing conditions, an increase of glutamic acid (Glu) has been detected in all fermentations, while concentrations of this amino acid decreased under oxidizing conditions (Table 9). Furthermore, γ-aminobutyric acid (GABA) increased under low redox potential, while it decreased under high redox potential in all fermentations (Table 9).

A multiple linear regression was performed to discover which environmental variables have an influence on FAA release. The used model explained ca. 63 % of variance (R^2= 0.6372). ANOVA displayed that both pH and ORP have a significantly influence on the FAA release during fermentations.

Analysis of volatile compounds was performed and reported in a heat map (Fig. 20). 1-hexanol, 2-butanone, acetic acid and benzaldehyde have been detected in all fermentations (Fig. 20). Ethanol was detected in all fermentations with *W. cibaria* and Mix culture under normal and reducing conditions only in fermentations with *W. cibaria* (data not shown). Changes of extracellular ORP affected the volatile compounds´ pattern of each fermentation (Fig. 20). *W. cibaria* produced under oxidizing conditions 1-propanol, 2-butanon-3-hydroxy, 2-heptanol, 2-propanone-1-hydroxy, while it produced 2-furanmethanol, 2-propanone-1-hydroxy, 3-methyl-3-buten-1-ol under reducing conditions. Moreover, 3-octanol was produced by *P. pentosaceus* under oxidizing status, while 2-furanmethanol, 1-butanol, 1-octen-3-ol and butyrolactone were produced under low ORP values. Besides, Mix culture produced 1-butanol, 2,3-butanedione, 2-butanone-3-hydroxy, 2-propanone-1-hydroxy, 3-octanol under oxidizing conditions, while, 1-propanol, 1-pentanol were produced under reducing conditions.

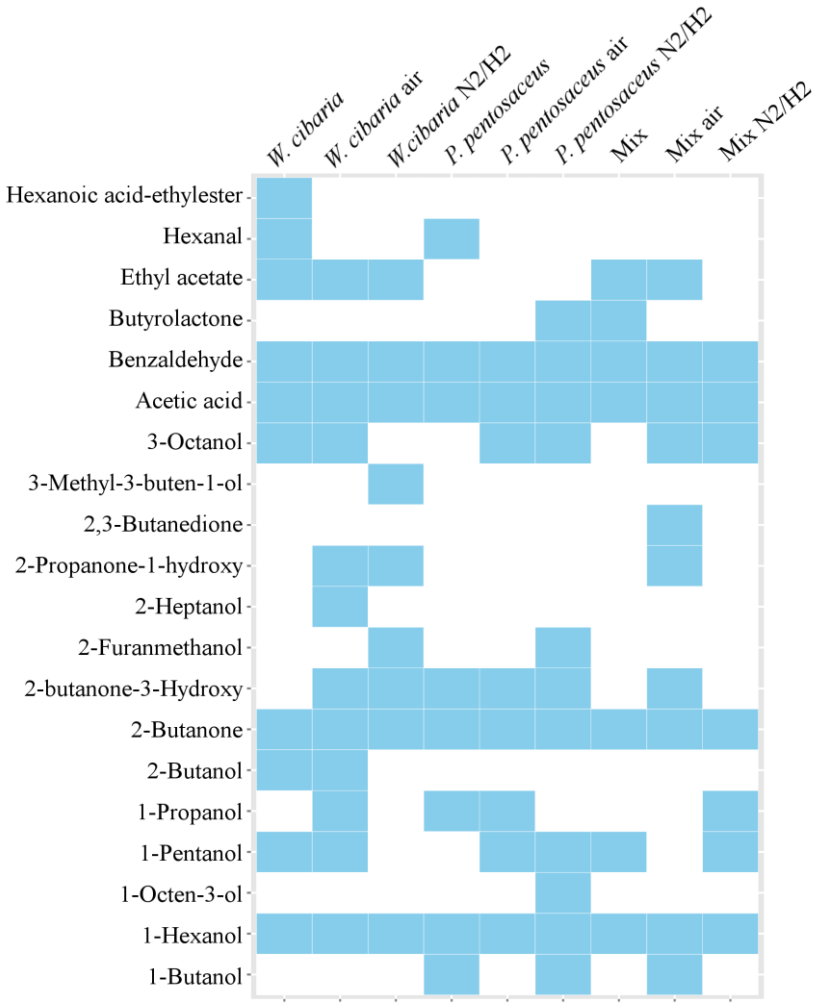

Fig. 20: volatile compounds' profile after 8 h fermentations (n = 3) with *W. cibaria* (TMW 2.1333), *P. pentosaceus* (TMW 2.6) and Mix culture.

3.4 Metabolic screening of starter culture in buckwheat SD

The goals of this investigation were to monitor microbial activity (observing many variables) of several heterofermentative (*hetero*), facultative heterofermentative (*f. hetero*) and homofermentative (*homo*) LAB in buckwheat SD. Indeed, nowadays less information is available about the LAB activity in GF SD, especially in buckwheat SD. Thus, this investigation may be helpful to discover this activity using multivariate statistical analysis.

34 variables were taken into account. FAN, free SH groups, pH, organic acids, ethanol and total FAA were consider as main variables for buckwheat SD. Moreover, carbohydrate consumption was detected and considered as well.

After fermentation, FAN content was not significant ($p < 0.05$) among the groups (Fig. 21a). Moreover, the proteolytic strain (*E. faecalis* TMW 2.630) did not display higher FAN content than other strains. *Hetero* showed a significant higher thiol content ($p < 0.05$) than *homo* and *f. hetero* (Fig. 21b). Furthermore, hetero produced low lactic acid and more acetic acid, showing significant low pH values compared to the other groups (Fig. 21c. e and f). Besides, *hetero* exhibited a significant ($p < 0.05$) higher FAA consumption than *f. hetero* LAB (Fig. 21d). *F. hetero* and *homo* mainly showed similar values among the variables, thus no significantly differences were observed ($p < 0.05$). Moreover, groups displayed outliers (Fig. 21c. d and f), indicating strain specific activities. Ethanol production was found only by *hetero*. Correlations between FAN, pH, thiol group and total FAA content were not detected. Acetic acid production was directly correlated with ethanol production ($R^2 = 0.86$) and with the increase of free SH groups ($R^2 = 0.44$) in fermentations with *hetero*. Furthermore, ethanol yield was directly linked with the increase of free thiols ($R^2 = 0.53$).

Inositol, rhamnose, arabinose, glucose, fructose, and sucrose were detected in fresh buckwheat doughs (before inoculation) and the total content was 19.18 ± 0.41 mM. Over fermentation time, mannitol, arabinose, and rhamnose were released, while other carbohydrates were consumed by LAB.

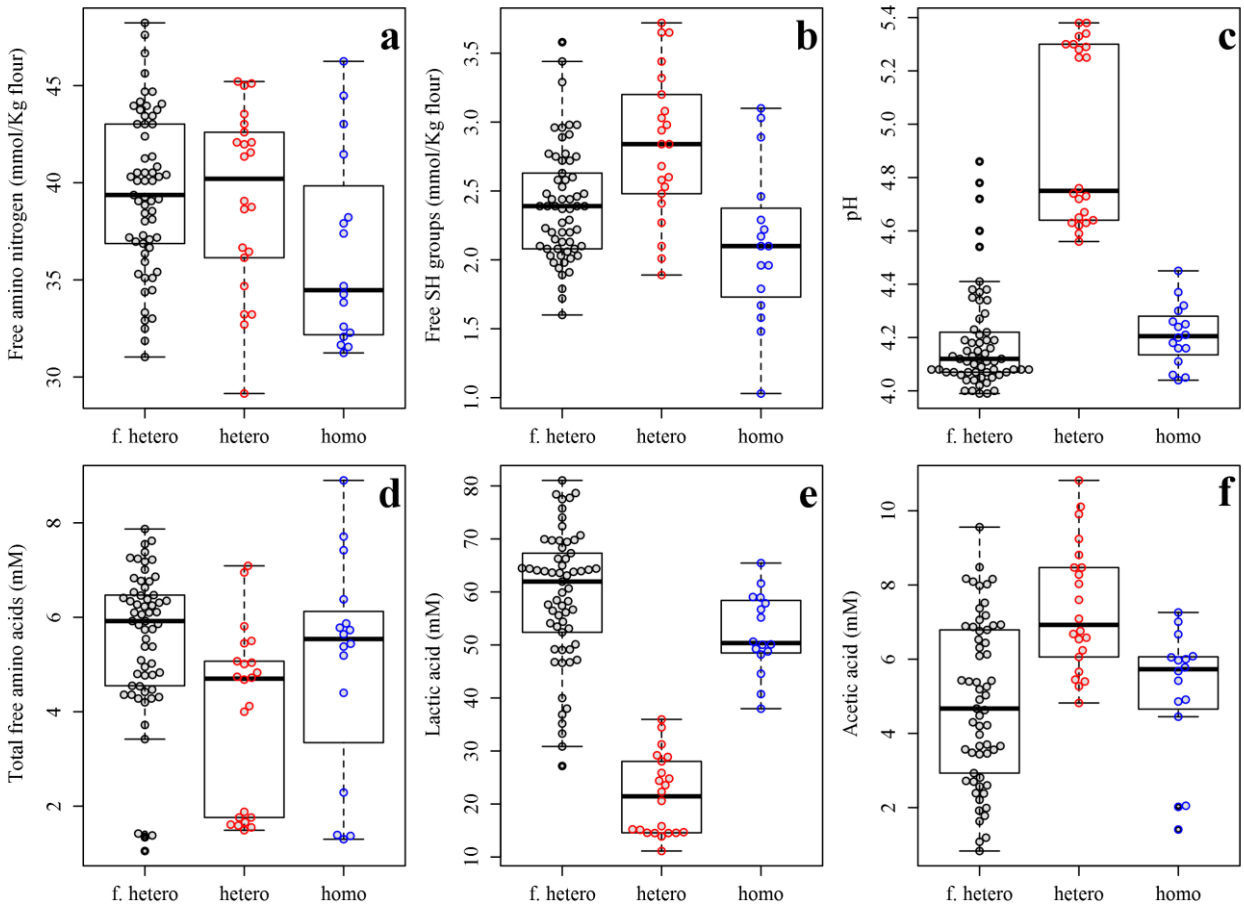

Fig. 21: Box plots of FAN (**a**), free SH groups (**b**), pH (**c**), FAA (**d**), lactic acid (**e**), acetic acid (**f**). Samples were grouped into: facultative heterofermentative (*f. hetero*), heterofermentative (*hetero*) and homofermentative (*homo*).

L. plantarum specie showed the highest total carbohydrate consumption (Fig. 22) but even *P. pentosaceus. W. cibaria*, and *W. confusa* species exhibited a high carbohydrate consumption (Fig. 22).

FAA content was reported in Fig. 23. Fresh dough showed a total FAA of 1.16 ± 0.13 mM, while after fermentation a higher FAA release was detected in almost each sample, except for some strains of *L. brevis*. *L. plantarum*, *L. mindensis*, and *L. brevis* strains did not use threonine, while the same behavior was monitored for alanine in fermentations with some strains of *L. paracasei*, *L. paralimentarius* and *L. sakei* (Fig. 23). *P. pentosaceus* and *W. cibaria* species were able to convert arginine to ornithine displaying a high yield production. However, even *L. brevis* strains were able to convert Arg to Orn but with low yield productions (Fig. 23).

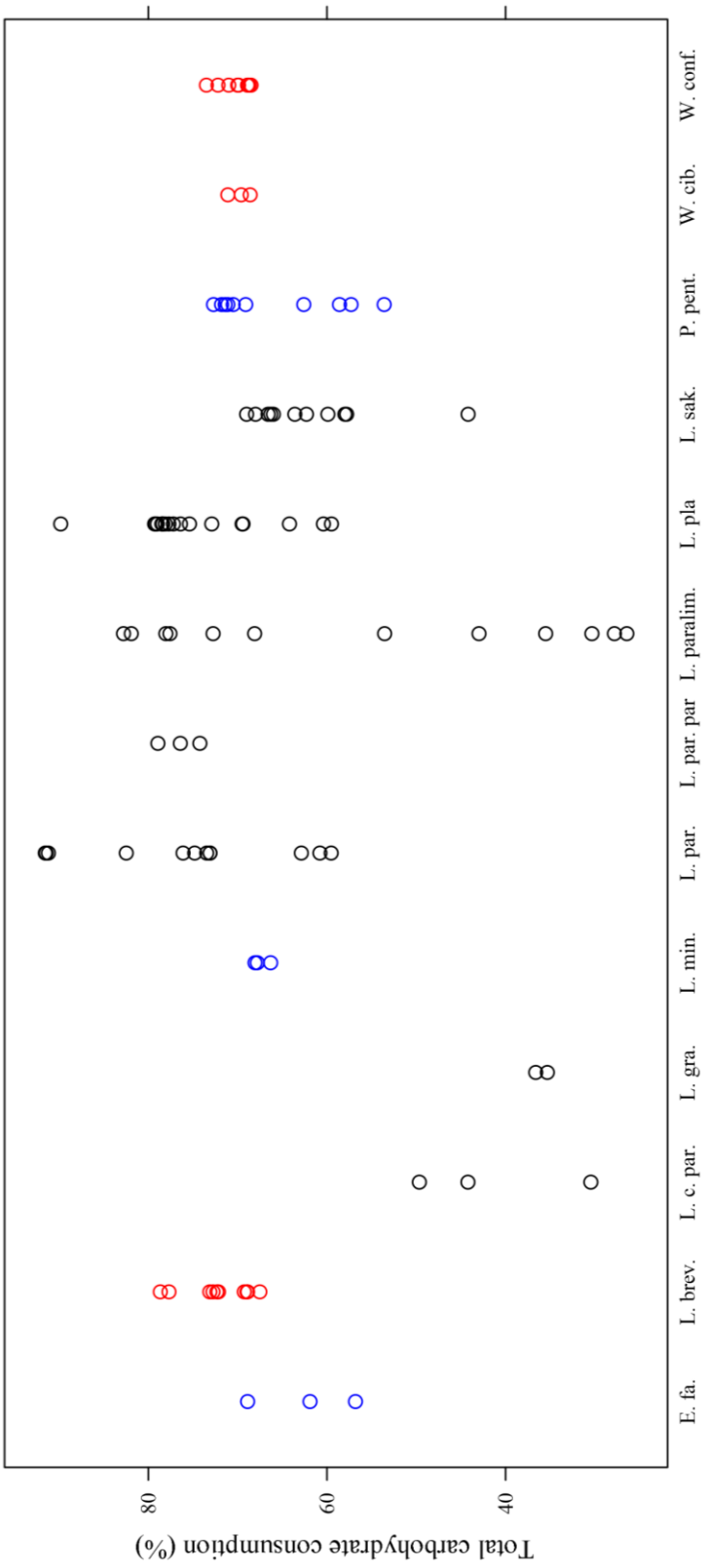

Fig. 22: total carbohydrate consumption in each species. Values were calculated subtracting the total carbohydrate content (after fermentation) from the initial total carbohydrate content of fresh dough. Colors indicate the metabolism type: blue (*homo*), red (*hetero*) and black (*f. hetero*).

Significance of LDA discrimination was tested by Monte Carlo test (each analysis was significant ($p < 0.01$)). The eigenvalues were tested by MANOVA and even in this case the analysis was significant ($p < 0.01$).

LDA (Fig. 24a) was performed to monitor main microbial activity (Fig. 24a) using 5 variables: FAN (ntest), free SH group content (sh), acetic acid concentration (acetate), lactic acid concentration (lactate) and total FAA content (AA). The eigenvalues of the discriminant axis were of 0.84 and 0.13, respectively. The first discriminant axis (LDA1) was represented by a separation of *hetero* from the other two groups (Fig. 24a). Moreover, the most important discriminating variables on the LDA1 were lactate and acetate. Furthermore, LDA2 was characterized by low separation between *homo* and *f. hetero*; the most important variables for the LDA2 were sh and ntest.

LDA of amino acid consumption was performed using 10 variables: His, Arg, Ala, Tyr, Val, Phe, Ile, Orn and Lys (Fig. 24b). Discrimination mainly occurred on the LDA1 (83.6 %) and the most important variable was Arg. In fact, the *f. hetero* was separated from *homo* and *hetero* (Fig. 24c). The most important discriminating variable of LDA2 (57.6%) was Orn. Indeed, *homo* was separated from *hetero* and *f. hetero* LAB. However, outliers which produced more Orn (*W. cibaria*) occurred in *hetero* group while outliers which produced less Orn (*L. mindensis*) occurred in *homo* groups (Fig. 24c).

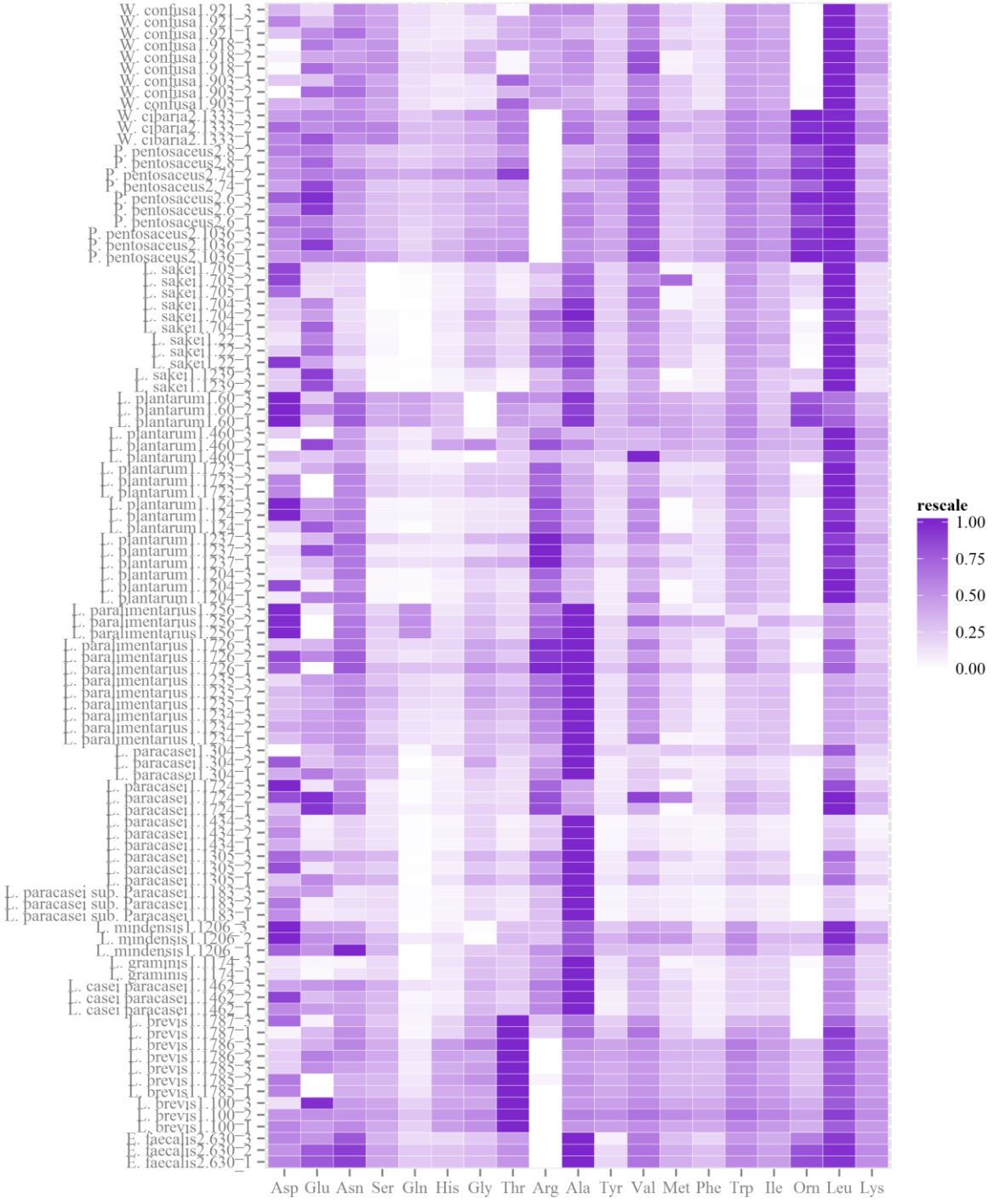

Fig. 23: Heat map of FAA in each replicate. Dark colors displayed a higher concentration while bright colors displayed low concentrations.

The LDA of carbohydrate consumption was performed using 6 variables: inositol, mannitol, rhamnose, fructose, glucose and arabinose (Fig. 24c). The discrimination occurred mainly on LDA1 (74.7 %) and the variables, which contributed the most to group separation, were arabinose and inositol. The LDA1 was represented by a separation of *hetero* from the other two groups. Moreover, fructose and glucose were the most important variables on the LDA2. *Homo* and *f. hetero* showed many similarities and both exhibited a wide spreading distribution of the points (Fig. 24c), i.e., points were more far away from confidential ellipses (outliers). LDA showed that employed LAB grouped well under the classification *homo*, *hetero* and *f. hetero*. However, outliers occurred and this means that some activities are more strain specific. This trend was well showed in Fig. 25, indeed colored patterns showed spread distribution in some variables.

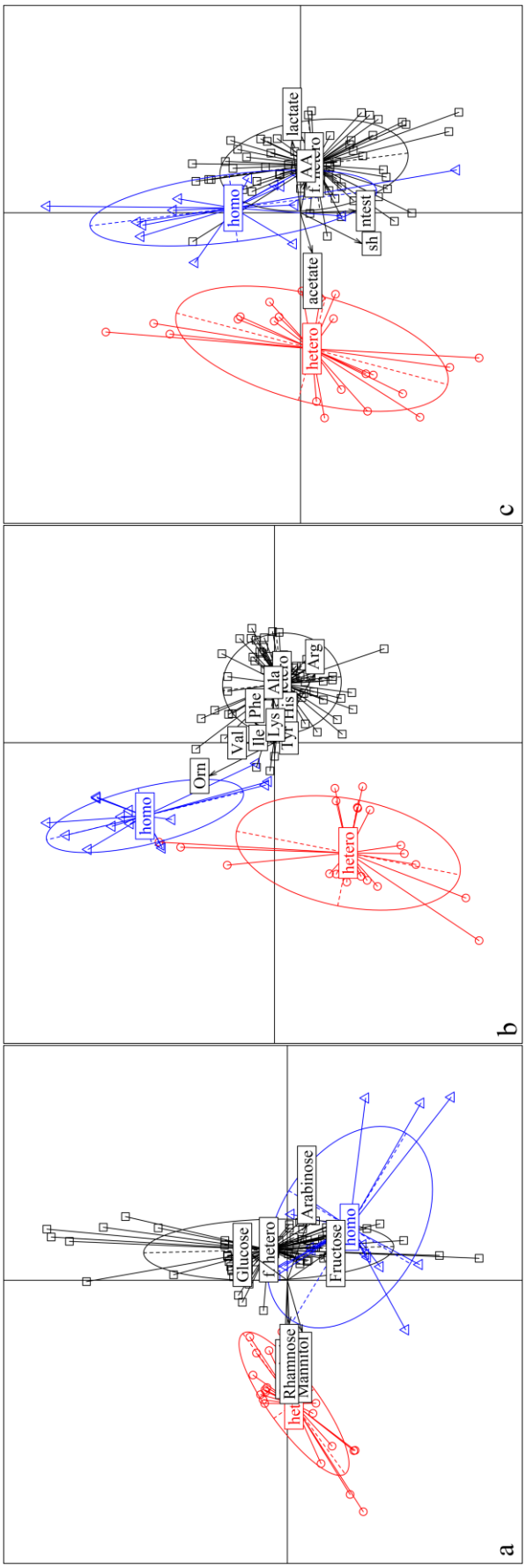

Fig. 24: LDA plots of carbohydrate metabolism (a), FAA (b), and microbial main metabolism (c). Arrows display variables' correlations. Metabolism type: blue (*homo*), red (*hetero*) and black (*f. hetero*).

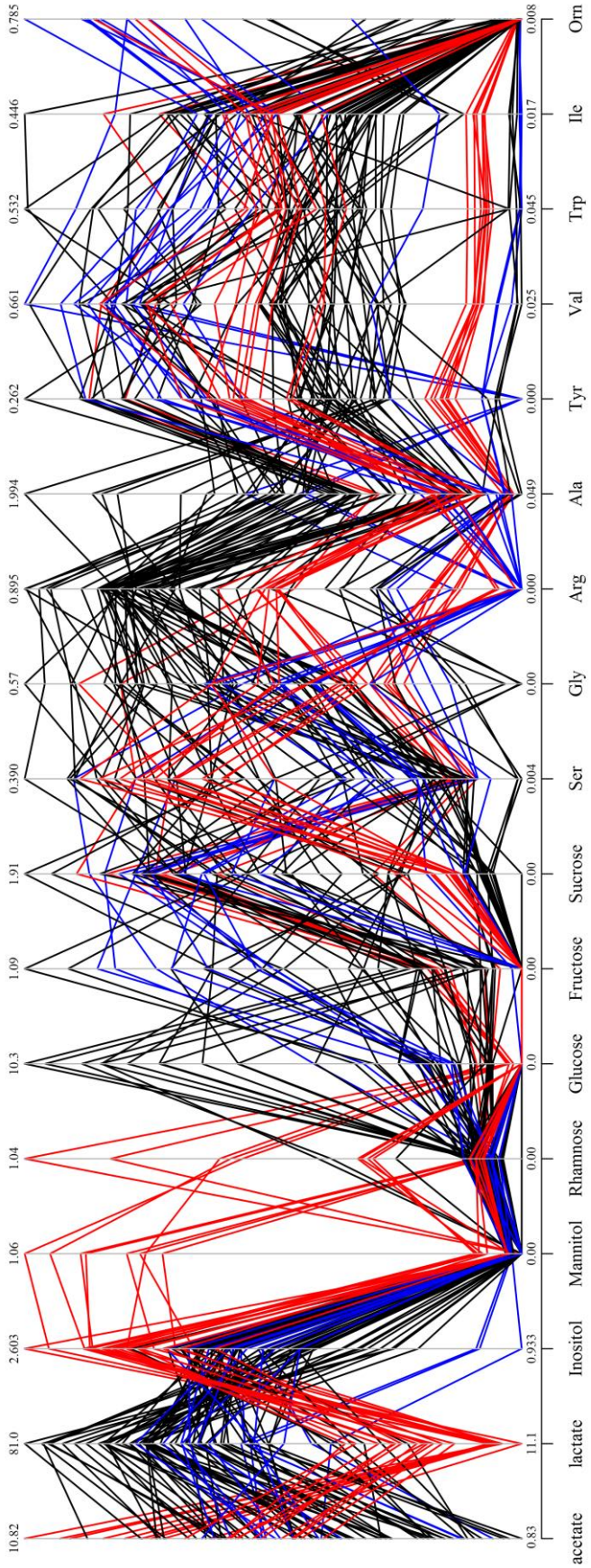

Fig. 25: parallel coordinates of the most important variables. Metabolism type: blue (*homo*), red (*hetero*) and black (*f. hetero*).

3.5 Effect of oxidizing and reducing buckwheat SD on brown rice and buckwheat batters and breads

To date, the effect of oxidizing and reducing SD with different fermentation times is still unknown. Nowadays, several scientific works about the effect of SD to GF breads are available (Galle et al., 2011; Houben et al., 2010; Hüttner et al., 2010; Moore et al., 2008, 2007; Moroni et al., 2011b; Rühmkorf et al., 2012). However, these works only specified a single microbial activity or omitted any microbial activity except the acidification. The goal of this investigation was to discover the effect of 'oxidizing' (containing *Pediococcus pentosaceus*) and 'reducing' (containing *Enterococcus faecalis*) buckwheat SD on buckwheat (BB) and brown rice (BR) bread and batter properties.

Cell counts were carried out in buckwheat SD. Each fermentation did not show contaminations and chemically acidified doughs did not display microbial growth (data not shown).

The pH of BB and BR batters (prepared with CA and SD) was reported in Table 10. BB and BR control batters (C and C-GSH) showed a pH of 6.20 ± 0.00, 6.14 ± 0.01 and 6.17 ± 0.03, 6.02 ± 0.03.

Table 10: pH values of buckwheat (BB) and brown rice (BR) batters prepared with CA and SD (8 and 24 h) fermented with *P. pentosaceus* (P) and *E. faecalis* (E)

Batter type	pH with pre-dough 8 h	pH with pre-dough 24 h
BB-CA	5.22 ± 0.04	5.03 ± 0.09
BB-P	4.96 ± 0.05	4.87 ± 0.05
BB-F	4.94 ± 0.00	4.67 ± 0.03
BR-CA	4.87 ± 0.07	4.51 ± 0.05
BR-P	4.61 ± 0.03	4.40 ± 0.01
BR-F	4.61 ± 0.06	4.29 ± 0.01

Frequency sweep tests were carried out on BB and BR batters. All batters showed a higher elastic modulus (G′) than viscous modulus (G″), indicating the solid elastic-like behavior of batters. BB batters displayed higher |G*| compared to BR batters, indicating that the BB batters have a high resistance to deformation. Significant low network strength (A_f) compared to BB batters was monitored in BR batters (Table 11). Moreover, BR batters displayed a higher significant ($p < 0.05$) network connectivity (z) in comparison with BB batters (Table 11). However, no significant differences ($p < 0.05$) were found for A_f and z

within BB batters as well as in BR batters. SD and CA of BB batters exhibited a higher δ than Control and GSH (C-GSH) doughs (Fig. 26a). BR batters showed high δ values in all samples starting from 10 Hz (Fig. 26b). However, significantly differences have not been monitored in the entire oscillatory range (Fig. 26b).

Table 11: Weak gel model of BB and BR batters. Values within columns with the same letter are not significant ($p < 0.05$). R^2 was > 0.95 for all samples.

Batter type	A_f	z	Batter type	A_f	z
BB-CA8	$2395^a \pm 23$	$6.17^a \pm 0.04$	BR-CA8	$1147^a \pm 241$	$6.42^a \pm 0.25$
BB-P8	$2282^a \pm 362$	$5.75^a \pm 0.23$	BR-P8	$703^a \pm 45$	$5.52^a \pm 0.15$
BB-F8	$2191^a \pm 128$	$5.86^a \pm 0.70$	BR-F8	$782^a \pm 145$	$6.48^a \pm 1.23$
BB-CA24	$2592^a \pm 183$	$5.71^a \pm 0.47$	BR-CA24	$1405^a \pm 412$	$7.94^a \pm 1.43$
BB-P24	$2084^a \pm 289$	$5.67^a \pm 0.20$	BR-P24	$1250^a \pm 453$	$5.89^a \pm 0.62$
BB-F24	$3019^a \pm 765$	$5.75^a \pm 0.36$	BR-F24	$732^a \pm 26$	$5.58^a \pm 0.15$
BB-Control	$2301^a \pm 111$	$5.96^a \pm 0.72$	BR-Control	$891^a \pm 101$	$6.11^a \pm 1.63$
BB-C-GSH	$2555^a \pm 341$	$5.97^a \pm 0.40$	BR-C-GSH	$940^a \pm 50$	$7.22^a \pm 0.20$

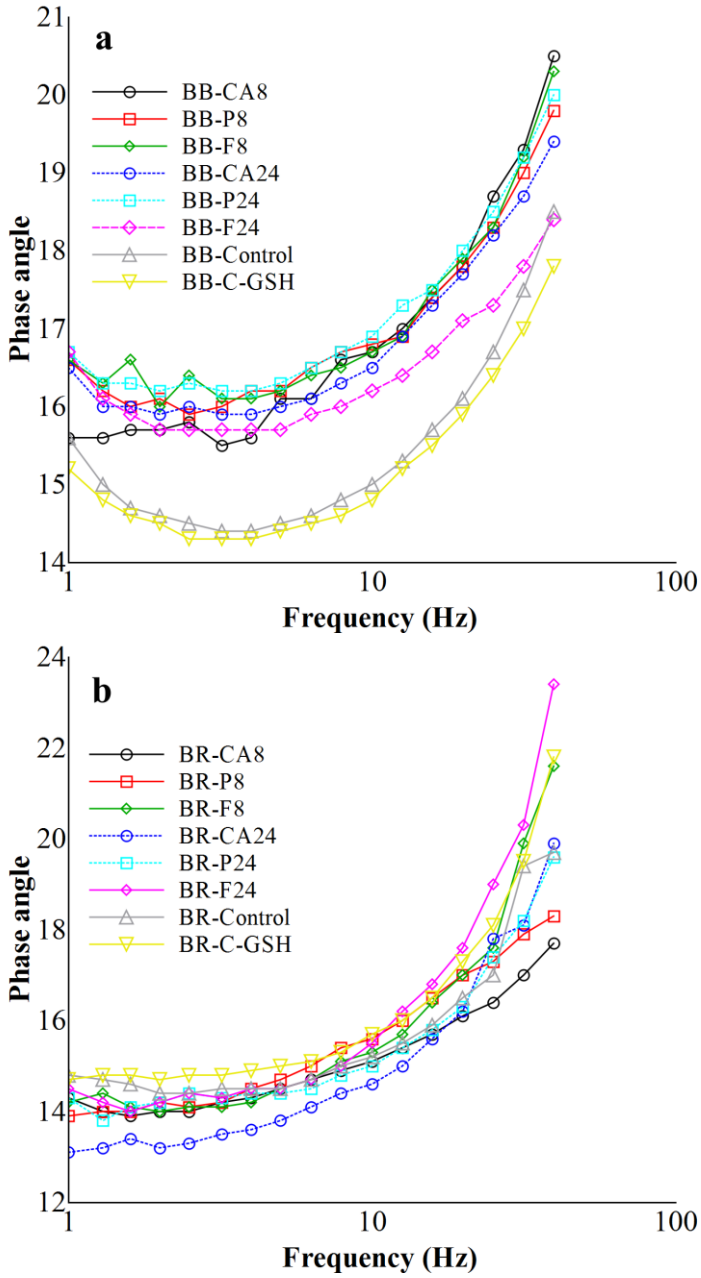

Fig. 26: Phase angle (δ) of BB (**a**) and BR (**b**) batters.

Temperature sweep tests were performed to monitor dough behaviors upon temperature changes. Because of dough drying, measurements were reported up to 6 min, as shown in Fig. 27. The high gelatinization peaks of BR batters were statistically significant ($p < 0.05$) compared to BB batters (Fig. 27a and b). Phase angle values did not show any notably differences in each BB and BR batters (data not shown). Maximal peaks have been detected between 78 and 80 °C in BB batters, as reported in Fig. 27a. All samples exhibited the same trend up to 2.5 min, but between 3.2 and 3.4 min different values of maximal peaks were observed (Fig. 27a). The highest peaks occurred in batters containing SD and CA doughs of

24 h. These peaks were statistically significant ($p < 0.05$) from Control and C-GSH batters (Fig. 27a). Similar gelatinization peaks between 75 and 80 °C have been detected in BR batters.

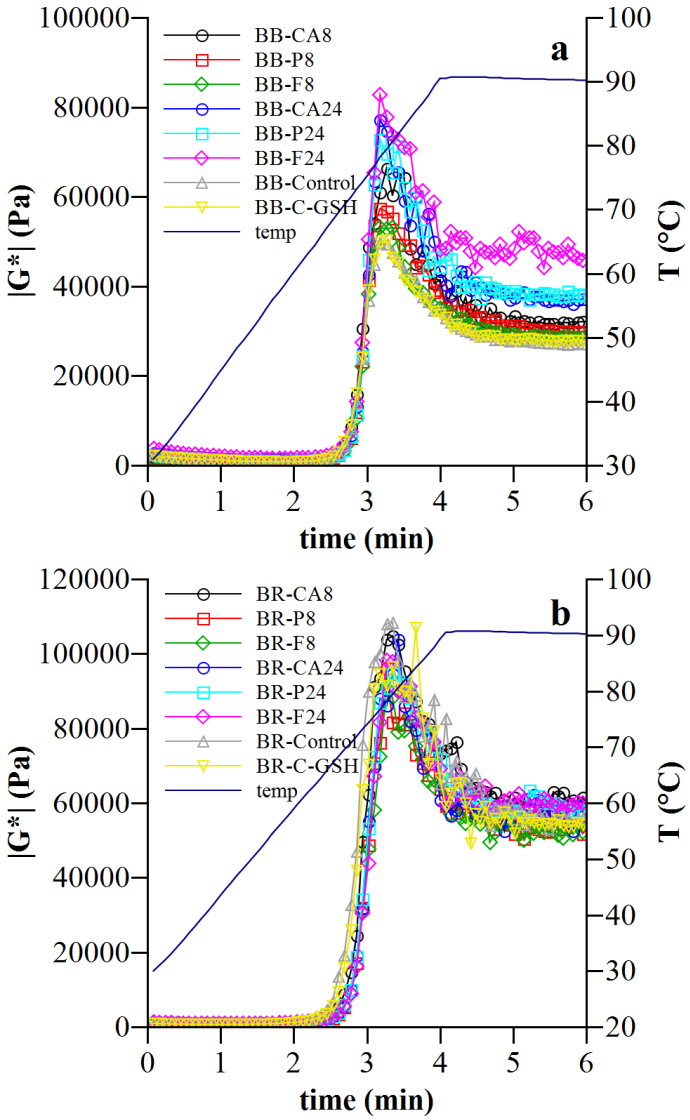

Fig. 27: Temperature sweeps at 1 Hz of BB (**a**) and BR (**b**) batters. Means are the average of three independent replicates.

Capillary gel electrophoresis before (BB-b and BR-b) and after (BBa and BRa) proofing was performed. Electrophoretic pattern of BB control batters (before and after proofing) was represented by proteins with a molecular weight (MW) of: 9, 15, 22, 30, 34, 38, 41 and 45 kDa. Proteins with a MW of 7, 14, 22, 31, 36, 38, 40, 45, 53 and 59 kDa have been detected in BR batters. Similar protein patterns of BB and BR batters were observed but BB did not display proteins with MW of 36 and 60 kDa (Fig. 28a,c). High protein content was found in BR batters due the high electropherogram intensity compared to BB control (data not shown).

Similar patterns were found in BB-b batters except in C-GSH batters, which showed high protein content between 4 and 20 kDa (Fig. 28a). After proofing (BB-a), proteolysis process has been observed in all samples especially in the range of 6.5 and 20 kDa. Moreover, BR-b and BR-a control samples showed higher protein content than other samples, except for F8 after proofing (Fig. 28d). After proofing, proteolysis occurred mainly in BR control samples (BR-a) than in samples containing extracts of CA and SD doughs (Fig. 28c,d).

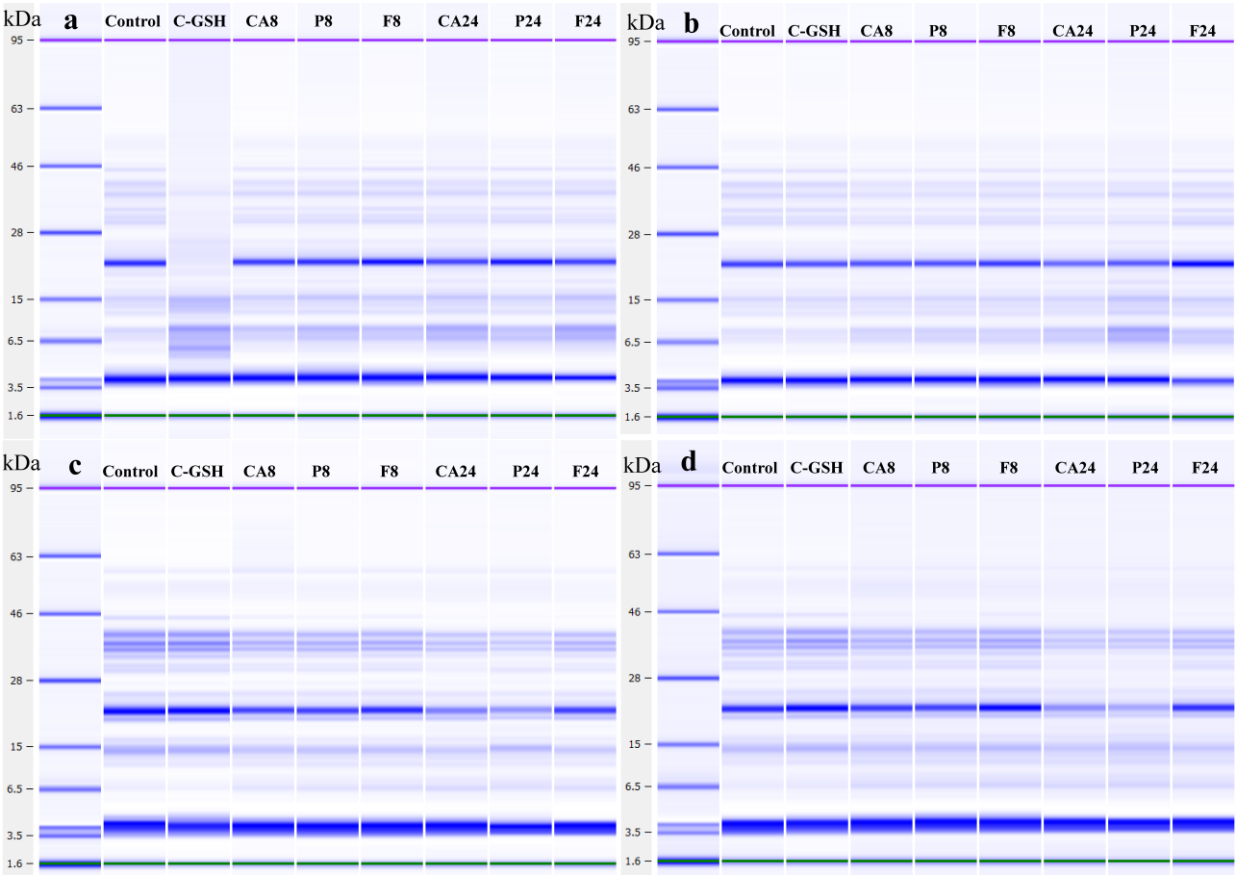

Fig. 28: electrophoretic pattern of BB (**a**, **b**) and BR (**c**, **d**) batter whole protein fraction before (**a**, **c**) and after (**b**, **d**) proofing. The first lane shows the ladder. Names above the lanes indicate the type of used pre-dough. Control: fresh buckwheat pre-dough; C-GSH: fresh buckwheat pre-dough containing 3 mM glutathione; CA: chemically acidified dough; P: sourdough fermented with *P. pentosaceus*; F: sourdough fermented with *E. faecalis*. Numbers beside letters indicate the fermentation time (h).

Significant ($p < 0.05$) higher volume and lower hardness values were found in BB breads compared to BR breads (Table 12 and Fig. 29). Significant differences on bread properties could not be detected in BB as well as in BR breads prepared with P and F sourdoughs (Table 12). The specific volume of BB breads in comparison with CA 8 h, Control, and C-GSH breads was significantly increased by the addition of 8 h SD (Table 12). The addition of 24

h CA and SD displayed a decrease of volume in BB breads (Table 12). Moreover, SD breads displayed a significant higher volume ($p < 0.05$) than control breads, except for F24 bread (Table 12). However, significant differences ($p < 0.05$) were not detected for volume in BR breads (Table 12).

Table 12: Volume, specific volume, TPA profile, and crumb properties of BB and BR breads prepared with C, C-GSH, CA, and sourdoughs. Values within columns in each bread group (BB and BR) with the same letter are not significant ($p < 0.05$).

Bread type	Volume (ml)	Crumb hardness (N)	Crumb cohesiveness	Crumb chewiness	Cell Diameter (mm)	Cell density (cell/mm^2)
BB-Control	$76.44^{a,b,c} \pm 1.35$	$8.48^{a} \pm 0.38$	$0.49^{a} \pm 0.02$	$3.37^{a} \pm 0.36$	$2.41^{a} \pm 0.14$	$0.53^{c} \pm 0.01$
BB-C-GSH	$77.44^{a,c} \pm 0.69$	$8.55^{a} \pm 0.45$	$0.47^{a} \pm 0.01$	$3.67^{a} \pm 0.38$	$2.32^{a} \pm 0.14$	$0.54^{c} \pm 0.01$
BB-CA 8h	$75.89^{a,b} \pm 0.51$	$8.11^{a,b} \pm 0.72$	$0.58^{a} \pm 0.06$	$3.81^{a} \pm 0.68$	$2.42^{a} \pm 0.10$	$0.46^{a,b} \pm 0.01$
BB-P 8h	$83.50^{e} \pm 0.87$	$5.77^{c,d} \pm 0.45$	$0.59^{a} \pm 0.05$	$3.37^{a} \pm 0.14$	$3.18^{b} \pm 0.23$	$0.43^{a,d} \pm 0.02$
BB-F 8h	$81.11^{d,e} \pm 0.19$	$6.26^{b,c,d} \pm 0.85$	$0.58^{a} \pm 0.06$	$3.55^{a} \pm 0.20$	$3.13^{b} \pm 0.05$	$0.40^{d} \pm 0.01$
BB-CA 24h	$74.11^{b} \pm 0.54$	$8.48^{a,b,d} \pm 0.04$	$0.56^{a} \pm 0.10$	$3.76^{a} \pm 0.26$	$2.17^{a} \pm 0.09$	$0.51^{b,c} \pm 0.01$
BB-P 24h	$80.33^{d} \pm 1.15$	$5.58^{c} \pm 0.54$	$0.54^{a} \pm 0.09$	$2.77^{a} \pm 0.57$	$3.06^{b} \pm 0.11$	$0.42^{a,d} \pm 0.03$
BB-F 24h	$78.78^{c,d} \pm 1.02$	$6.21^{c,d} \pm 0.55$	$0.54^{a} \pm 0.09$	$3.34^{a} \pm 0.33$	$2.93^{b} \pm 0.08$	$0.43^{a,d} \pm 0.02$
BR-Control	$67.56^{a} \pm 1.71$	$7.91^{d} \pm 0.58$	$0.40^{a} \pm 0.02$	$2.85^{a} \pm 0.01$	$2.41^{a,b} \pm 0.14$	$0.53^{a,b} \pm 0.01$
BR-C-GSH	$68.33^{a} \pm 1.15$	$8.84^{b,d} \pm 0.29$	$0.40^{a} \pm 0.01$	$3.21^{a,b} \pm 0.18$	$2.32^{a,b,c} \pm 0.14$	$0.54^{a,b,c} \pm 0.01$
BR-CA 8h	$65.00^{a} \pm 2.52$	$11.63^{a,b,c} \pm 1.13$	$0.40^{a} \pm 0.02$	$4.09^{a,b} \pm 0.34$	$2.60^{a,b,c} \pm 0.17$	$0.52^{a,b,c} \pm 0.03$
BR-P 8h	$69.89^{a} \pm 1.35$	$11.54^{a,b,c} \pm 1.19$	$0.45^{b,c} \pm 0.01$	$4.73^{b} \pm 0.40$	$3.18^{b,c} \pm 0.32$	$0.50^{a,b,c} \pm 0.02$
BR-F 8h	$69.56^{a} \pm 0.96$	$11.72^{a,c} \pm 0.87$	$0.47^{b} \pm 0.01$	$4.53^{b} \pm 1.21$	$3.30^{c} \pm 0.16$	$0.48^{b,c} \pm 0.02$
BR-CA 24h	$57.56^{b} \pm 3.50$	$13.62^{c} \pm 1.63$	$0.38^{a} \pm 0.03$	$4.03^{a,b} \pm 0.70$	$2.09^{a} \pm 0.34$	$0.61^{a} \pm 0.08$
BR-P 24h	$68.00^{a} \pm 3.21$	$9.51^{a,b,d} \pm 0.93$	$0.41^{a,c} \pm 0.01$	$3.50^{a,b} \pm 0.21$	$3.01^{b,c} \pm 0.35$	$0.47^{c} \pm 0.03$
BR-F 24h	$66.78^{a} \pm 2.12$	$10.03^{a,b,d} \pm 0.74$	$0.42^{a,b,c} \pm 0.00$	$3.86^{a,b} \pm 0.19$	$2.59^{a,b,c} \pm 0.37$	$0.49^{b,c} \pm 0.02$

BB sourdough breads displayed softer crumb hardness than control breads. However, even in this case significant differences could not be detected between the two SD breads (P and F) (Table 12). Whereas, CA and SD BR breads exhibited high crumb hardness and cohesiveness compared to Control and C-GSH breads (Table 12). Moreover, the use of 24 h sourdoughs showed a decrease (statistically insignificant) of crumb hardness, chewiness, and cohesiveness in BR breads, as reported in Table 12. Well distributed cells compared to BR breads have been monitored in BB bread slices, especially in sourdough breads (Fig. 29). Slices of sourdough BB showed bigger cells than control breads, especially for those prepared using 8 h SD (Fig. 29a). Moreover, this behavior has been confirmed by C-Cell results (Table 12). Slices of BR breads displayed higher cell diameter than control breads (Fig. 29b), however it was not statistically significant ($p < 0.05$) (Table 12).

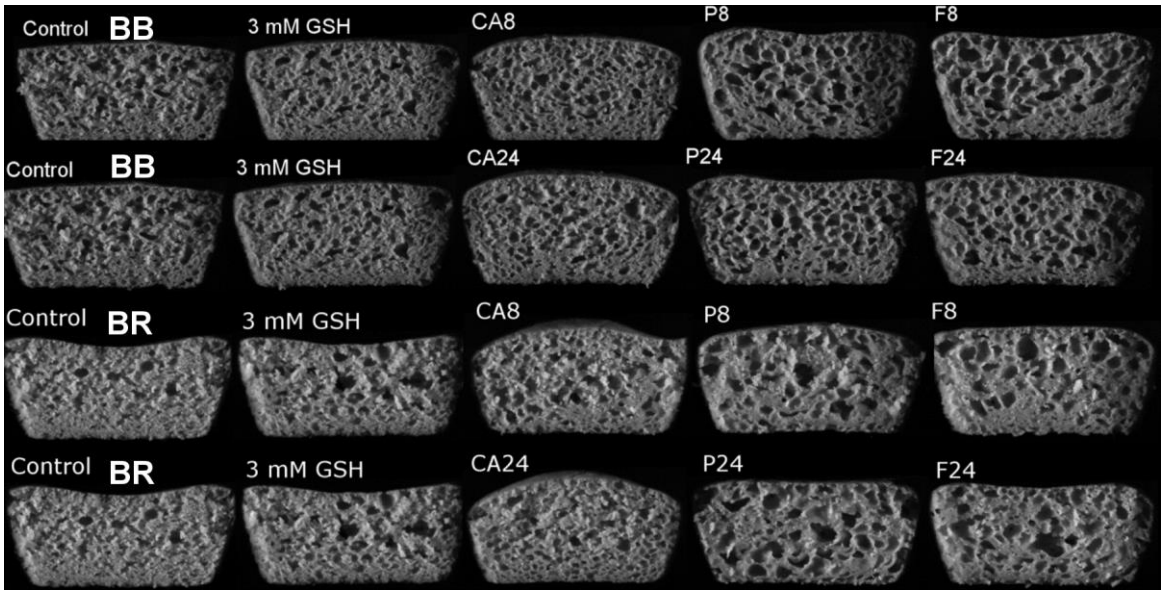

Fig. 29: Bread slices of BB (**a**) and BR (**b**) breads. Control: fresh buckwheat pre-dough; C-GSH: fresh buckwheat pre-dough containing 3 mM glutathione; CA: chemically acidified dough; P: sourdough fermented with *P. pentosaceus*; F: sourdough fermented with *E. faecalis*. Numbers beside letters indicate the fermentation time (h).

Significant linear correlations were not detected between batter rheology and bread properties in BB as well as in BR trials. The volume was correlated with hardness, cell diameter, cell density as well as pH of the batters in BB breads (Fig. 30a). Whereas similar correlations between hardness, volume and cell diameter could be detected in BR breads (Fig. 30b).

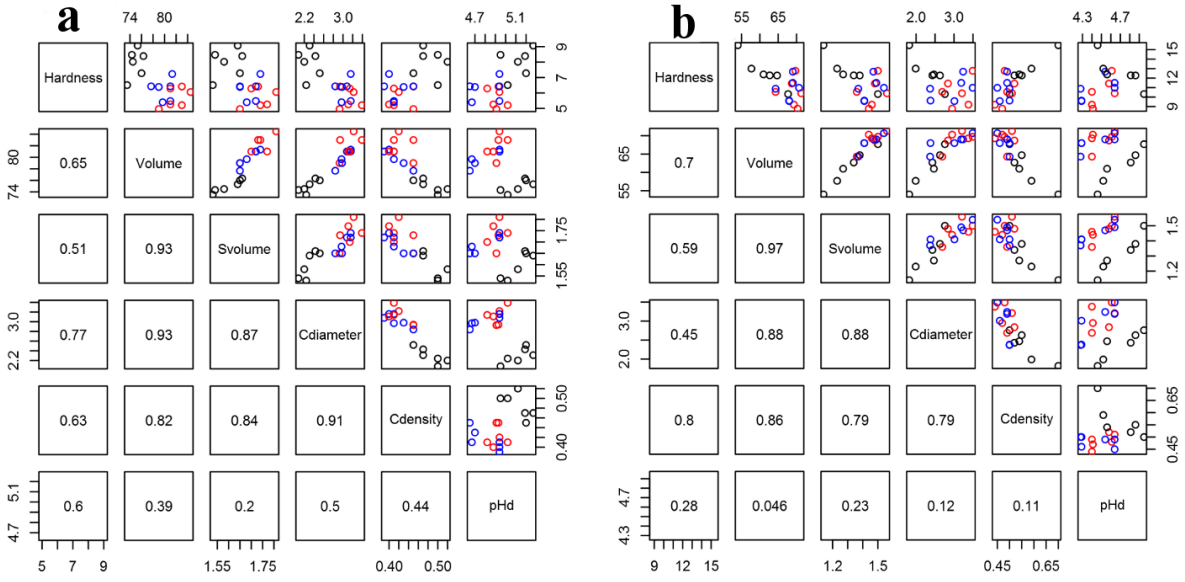

Fig. 30: Scatter plot matrix of BB (a) and BR (b) breads of following variables: hardness, volume, specific volume (Svolume), cell diameter (Cdiameter), cell density (Cdensity) and pH of batters (pHd). Colors indicates the type of pre-dough used for baking: CA (black), P (red) and F (blue). The lower panel displays the Pearson' correlation coefficients.

3.6 Influence of different SD and dry yeasts on GF bread characteristics

In section 3.5, it was mentioned that the SD can influence the yeast activity during the proofing time. To proof this effect, many baking trials were performed using three different commercial dry yeasts (Pante red, Fermipan rot and Lallemand) and 5 different SD. The SD were prepared with 5 different mixed starter cultures: LAB1 (*Lactobacillus mindensis* TMW 1.1206 + *Lactobacillus brevis* TMW 1.305), LAB2 (*Lactobacillus plantarum* TMW 1.1723 + *Lactobacillus paracasei* 1.1724), LAB3 (*P. pentosaceus* 2.6 + *L. paracasei* 1.1305), LAB4 (*Lactobacillus paracasei* 1.1305 + *Lactobacillus brevis* 1.1786) and LAB5 (*Pediococcus pentosaceus* TMW 2.6 + *Lactobacillus mindensis* TMW 1.1206).

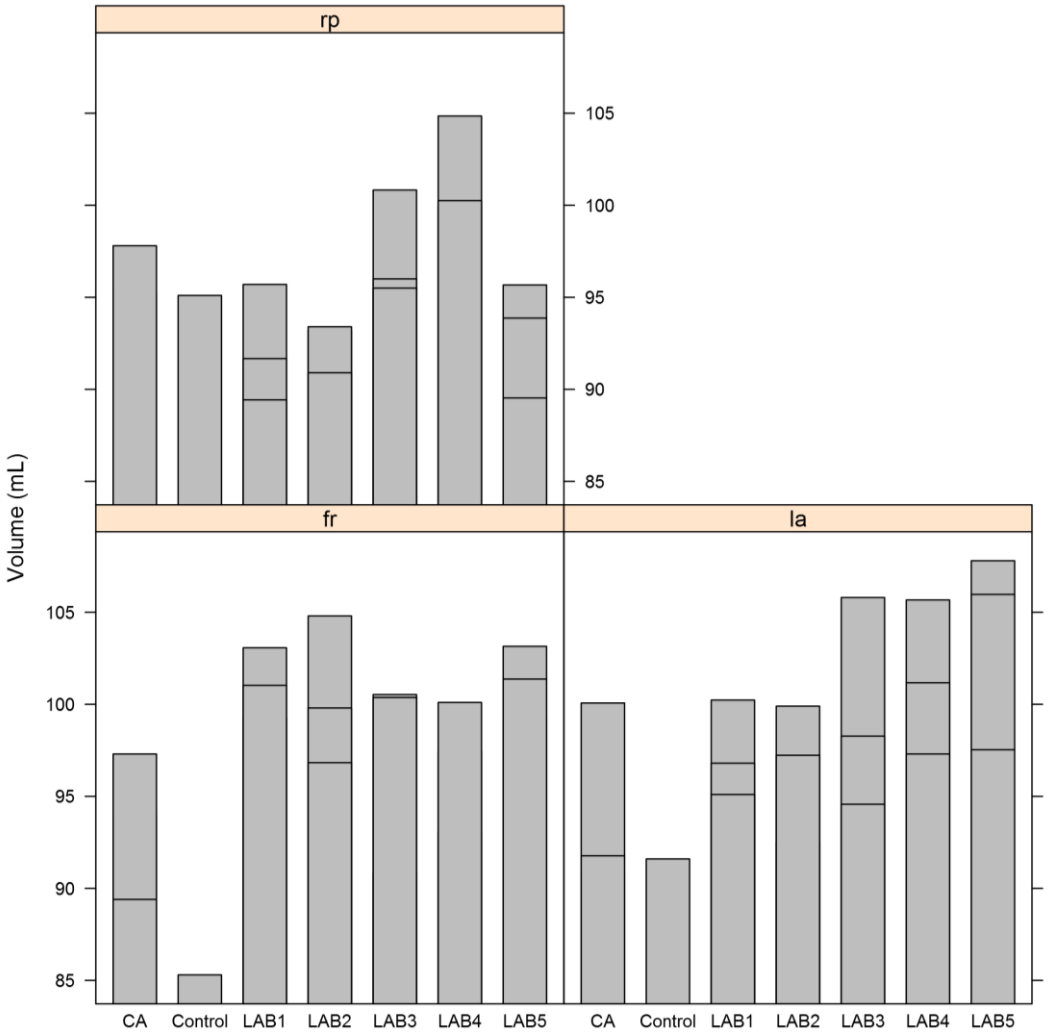

Fig. 31: Bar plot volume of buckwheat bread prepared with CA (chemically acidified dough), Control (fresh buckwheat pre-dough), LAB1 (*L. mindensis* TMW 1.1206 + *L. brevis* TMW 1.305), LAB2 (*L. plantarum* TMW 1.1723 + *L. paracasei* 1.1724), LAB3 (*P. pentosaceus* 2.6 + *L. paracasei* 1.1305), LAB4 (*L. paracasei* 1.1305 + *L. brevis* 1.1786) and LAB5 (*P. pentosaceus* TMW 2.6 + *L. mindensis* TMW 1.1206). Each trial was performed using three different dry yeast: rp (Pante red), fr (Fermipan rot) and la (Lallemand).

These co-cultures were employed to stay close to the practical applications, where co-cultures are often used instead of single culture due to contamination risks. Moreover, co-cultures increase the complexity of fermentations and this can lead to difficult understanding of molecular activities. Nevertheless, this complexity is very helpful to extend the probability to obtain more different interactions between LAB and yeasts, and this could be difficult only using three type of strains, homofermentative, heterofermentative and facultative heterofermentative. The co-cultures' combinations were chosen considering the FAA consumption and reducing activity of the strains. These two factors were taken into account due to their possible influence on yeast activity. We summarized the cocultures properties as follow:

- LAB1: high FAA consumption (*L. mindensis*) and high reduction (*L. brevis*):
- LAB2: high reducing activity of both strains (*L. plantarum* and *L. paracasei*)
- LAB3: low reducing activity (*P. pentosaceus*) and low FAA consumption (*L. paracasei*)
- LAB4: high reducing activity (*L. paracasei*) and high FAA consumption (*L. brevis*)
- LAB5: both strains show low reducing activity (*P. pentosaceus* and *L. mindensis*)

Bread volumes of trials prepared with different dry yeasts and SD are displayed in Fig. 31. A clear volume increase was observed in trials with SD. However, rp (Pante red) trials showed the lowest volume's values compared to the other trials. Indeed, breads prepared with LAB2 and LAB5 exhibited a low volume compared to the control, as also shown in Fig. 31 and Fig. 32. The clear volume increase through SD addition is also well displayed in Fig. 32. Moreover, it was observed that, depending on the combination yeast – SD, the bread volume changed differently (Fig. 32), thus even the gas production.

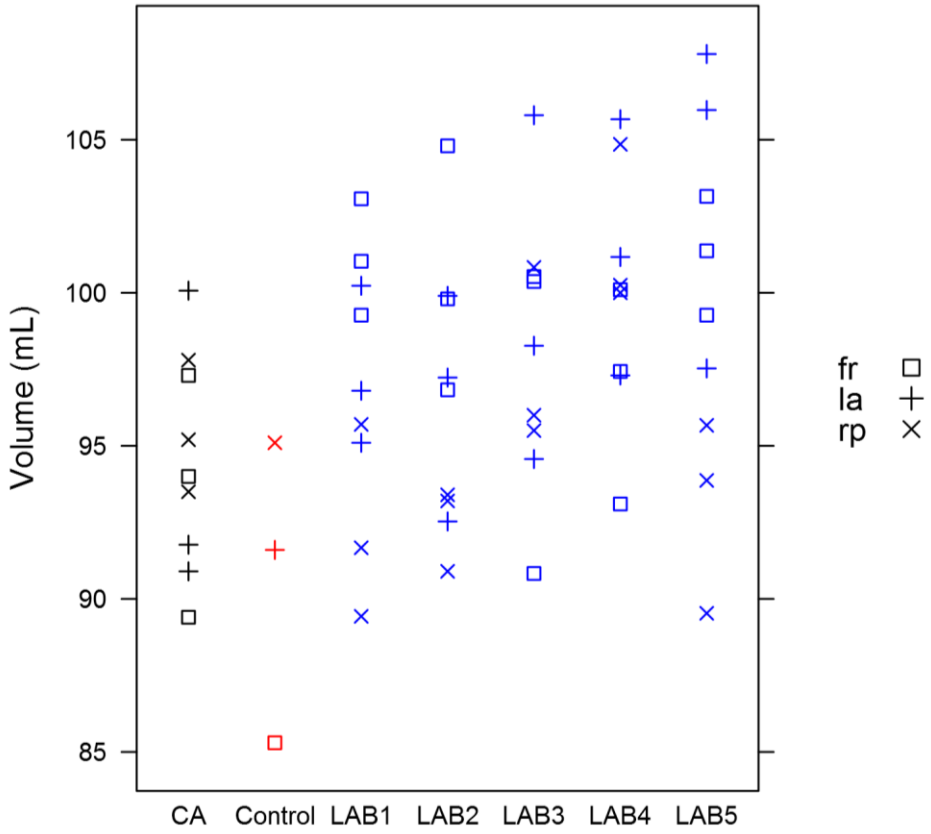

Fig. 32: Strip plot of volume of buckwheat bread prepared with CA (chemically acidified dough), Control (fresh buckwheat pre-dough), LAB1 (*L. mindensis* TMW 1.1206 + *L. brevis* TMW 1.305), LAB2 (*L. plantarum* TMW 1.1723 + *L. paracasei* 1.1724), LAB3 (*P. pentosaceus* 2.6 + *L. paracasei* 1.1305), LAB4 (*L. paracasei* 1.1305 + *L. brevis* 1.1786) and LAB5 (*P. pentosaceus* TMW 2.6 + *L. mindensis* TMW 1.1206). Each trial was performed using three different dry yeast: rp (Pante red), fr (Fermipan rot) and la (Lallemand).

The volume increase (Fig. 33) caused by SD addition was statistically significant, as shown by ANOVA. ANOVA indicated that the application of the three different yeasts did not show any significant effect on CA bread volume. However, ANOVA showed that the addition of different pre-doughs had a significant influence on bread volume. Moreover, bread prepared with CA displayed an increasing trend on bread volume compared to the control (Fig. 33). However, this trend was not statistically significant. Among the SD breads, the addition of SD LAB4 exhibited the best results considering all trials (with the three yeasts). Indeed, LAB4 showed the highest volume, 99.98 mL ± 3.85, with the lowest standard deviation compared to the other SD breads.

As mentioned previously in section 3.4, even in this case the bread volume was indirectly correlated with the crumb hardness (Fig. 34), i.e., breads with high volume were softer than bread with low volumes. Even in these tests, it could not be detected any clear correlations between bread properties (volume and hardness) and dough rheology (Fig. 34).

Other than these parameters, proteolysis processes and free thiol content of the employed SD were taken into account. However, no correlations were detected between these parameters and the bread properties. Thus, proteolysis and free thiol content changes did not affect the properties of buckwheat breads.

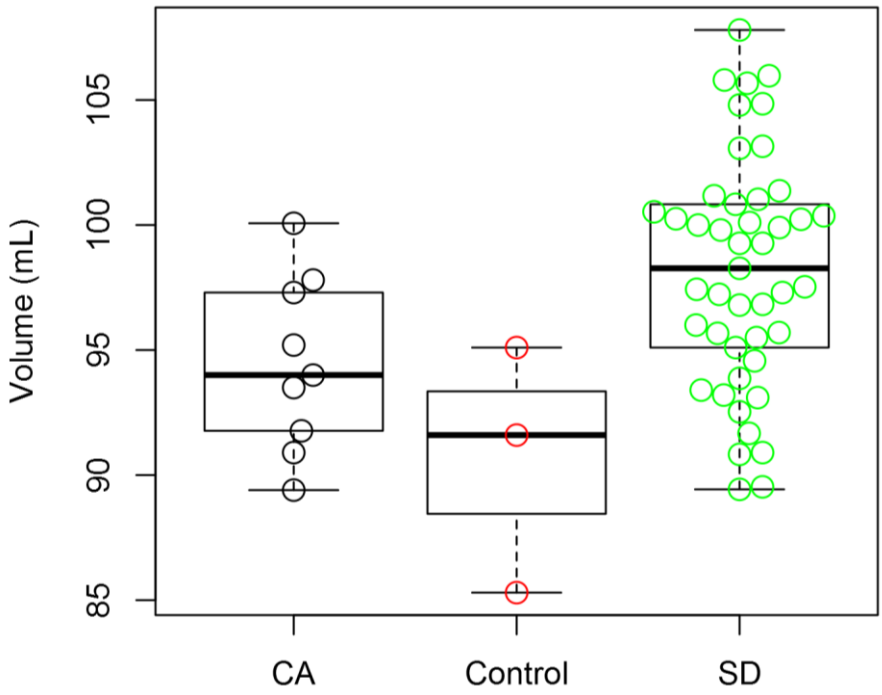

Fig. 33: box plots of volume of buckwheat breads prepared with CA (chemically acidified dough), Control (fresh buckwheat pre-dough) and SD (all SD included). In the box plots are also included the trials with the three different dry yeasts.

The pH of the dough, 5.6-5.2 and 5.8-5.3, did not influence Lallemand and Fermipan rot yeasts. Instead, dough pH (5.7-5.4) influenced significantly the activity of Pante red yeast, as also shown by ANOVA. Moreover, breads prepared with LAB2 and LAB5 showed the lowest pH of the dough compared to the other SD breads.

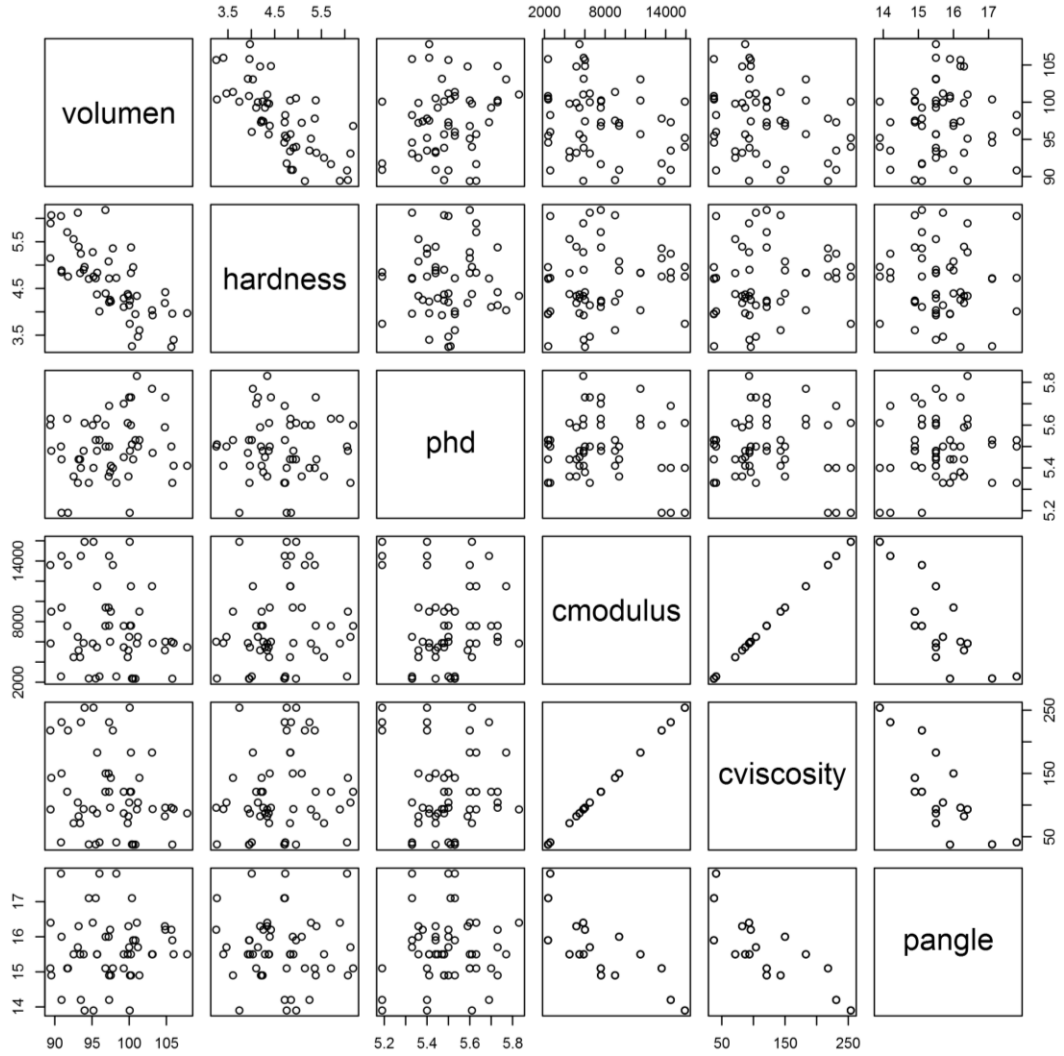

Fig. 34: correlation matrix between bread characteristics (volume (mL) and hardness (N)) and dough rheology (complex modulus (cmodulus), complex viscosity (cviscosity), and phase angle (pangle)).

4 Discussion

In this work, for the first time, ORP measurements were performed in sourdough system, showing a high reproducibility of this method. It has been shown that the LAB used had a reducing activity during buckwheat sourdough fermentations. The changes in ORP were correlated with the free thiol content of buckwheat SDS-soluble fraction. However, the LAB reducing activity did not improve the proteolysis as expected but the addition of reducing agents, such as glutathione, decreased the proteolytic process in buckwheat sourdough. The employed strains did not show proteolytic activity, which was mainly caused by flour endogenous proteases.

In the experiments, it was also observed that oxidizing conditions increased the number of volatile compounds, while under reducing conditions the number of volatile compounds decreased. Moreover, LAB showed a strain specific metabolism in buckwheat sourdoughs. Furthermore, 'reducing' and 'oxidizing' sourdoughs did not affect the properties of GF breads. However, sourdoughs demonstrated an increase of GF bread volume. Therefore, a positive impact of these bacteria on the metabolic activity, namely gas formation, of the yeasts was suggested.

All these results are deeply discussed in the next section.

4.1 Influence of LAB growth on ORP in buckwheat SD

The influence of lactic acid bacteria and their metabolism on the ORP was demonstrated using redox potential measurements during buckwheat sourdough fermentations.

Results of section 3.1 reported that the reducing activity of LAB in sourdoughs was comparable to the those monitored in other food matrix, e.g., milk, cucumber, and cheese (Abraham et al., 2007; Cachon et al., 2002; Olsen and Pérez-Díaz, 2009). The ORP does not only indicate the level of DO (Kjaergaard, 1977), thus, considering a constant temperature, the initial ORP of doughs is also affected by the presence of other reducing and oxidizing compounds. Differences of ORP time courses related to LAB main metabolism, homo- (*L. plantarum* TMW 1.460, *L. sakei* TMW 1.22 and *P. pentosaceus* TMW 2.6) or heterolactic fermentation (*W. cibaria* TMW 2.1333), were not detected among the strains.

The maximal acidification rate (V^a_m) occurred only after the reducing step in all fermentations (Fig. 9). It could be linked to the influence of ORP changes on microbial metabolic flux, as even reported in fermentations with *Escherichia coli* (Riondet et al.,

2000). Moreover, low ORP values (reducing conditions) could increase a high metabolites' production or fast acidification times in fermentations with LAB (Ji-dai et al., 2008).

A slight reduction of the acidification rate occurred after ca. one hour (Fig. 9b,c and d). In this case, the media were first inoculated with high cell density, showing high metabolic activity, but afterwards the starter culture needed most probably time for medium adaptation and therefore the slight decrease of acidification rate occurs.

P. pentosaceus (TMW 2.6) showed high ORP compared to *L. plantarum* (TMW 1.460) (Fig. 5) and this behavior was also observed by Brasca et al. (2007) in milk fermentations. The big difference in reducing activity between *W. cibaria* (TMW 2.1333) and *P. pentosaceus* (TMW 2.6) could be related either to the different growth or to the different metabolism, which the two strains showed (Fig. 5, Fig. 6 and Table 4).

Even though only four LAB strains were used for this investigation, the results indicated that each strain has typical redox potential time course (always depending on the inoculation concentration) in buckwheat SD fermentations. This trend was even observed by Brasca et al., (2007) in milk fermentations. According to these results, redox potential measurements could be capable of LAB discrimination even in different sourdough types. Moreover, since the ORP is strain specific, it could be employed to predict the presence of contamination in SD fermentations, as also showed in other research works (Olsen and Pérez-Díaz, 2009). Thus, ORP measurements can be used as quality parameter during sourdough fermentation in the industrial production.

The use of appropriate starter culture (reducing or oxidizing) could be theoretically decisive for structural quality of GF bakery products. Indeed, in the wheat system, different ORP states affect the protein structure and finally dough rheology and thus bread properties (Jänsch et al., 2007). Moreover, other studies have demonstrated that extracellular ORP can affect the aroma formation in fermentation with LAB (Kieronczyk et al., 2006). This aspect was discussed in detail in section 4.5. These results show the high reproducibility of ORP measurements in SD fermentations and the same reducing capacity of LAB like in other media has also been observed in buckwheat SD fermentations.

4.2 Influence of LAB on redox status and proteolysis of buckwheat SD

The results showed the influence of LAB growth on extracellular redox status and proteolysis in buckwheat SD.

The redox status was measured using ORP measurements and by detection of free thiol content of buckwheat SDS-soluble fraction. It should be considered that, the ORP drop does not only depend on free SH group content but it is also linked to the oxygen reduction and to the presence of several redox compounds. Hence, non-inoculated doughs, such as CA and CA containing glutathione, displayed a more oxidized status than fermented doughs. Indeed, oxygen reduction has not been detected. However, CA doughs containing GSH were more reducing than CA due to the presence of glutathione. Fermentations with *P. pentosaceus* (TMW 2.6) displayed a fast decrease of thiol content during the active phase. This phenomenon could be related to the consumption of low-molecular weight compounds but it could be also linked to the oxidative status, in which disulfide bound formation normally occurs. However, fermentations with *E. faecalis* (TMW 2.630) and *W. cibaria* (TMW 2.1333) showed high thiol contents after 8 h fermentations. This increase could be related to high reduction activity of these two strains, which probably reduced disulfide bounds. Still, after 24 h, a low thiol content was observed in each fermentation. Indeed, ORP started to decrease after 8 h, where used strains showed a decrease of microbial activity which couldn't contrast the oxidation activity of oxygen. Thus, an extended oxidation of free thiols into disulfide bounds occurred probably in each fermentation.

The results of this investigation demonstrated the presence of several endogenous proteases in buckwheat dough, e.g., metalloproteases, serine, aspartic, and cysteine proteases. These results are also in agreement with other studies (Dunaevsky and Belozersky, 1998, 1989; Timotijevic et al., 2003). However, no one of these specific proteases showed a predominant activity in sterile doughs. Probably, aspartic proteases have a major activity during acidification down to pH 3.3, always considering the rising trend we detected in the test with sterile doughs. Aspartic proteases showed an optimum activity at pH 3.1, while metalloproteases, cysteine, and serine proteases displayed their optimum at pH 9, 5.5 and 8.2 (Dunaevsky and Belozersky, 1989; Dunaevsky et al., 1998; Timotijevic et al., 2003). Considering this information, it could be hypothesized that cysteine proteases played a main role upon the first acidification step (up to pH 5.5) and afterwards aspartic proteases started to play a major role on proteolysis during the second part of fermentation (down to pH 3.1).

Observing the results about FAN content of SD fermentations, proteolysis was mainly caused by buckwheat flour endogenous proteases. Indeed, CA doughs, which did not show any microbial growth, exhibited a high FAN content compared to the SD after 8 h fermentation. Moreover, since the used strains, except *E. faecalis* (TMW 2.630) did not show own proteolytic activity, proteolysis can only be caused by the endogenous proteases contained in buckwheat flour. Surely, endogenous proteases could also be influenced indirectly by microbial activity, such as acidification. Furthermore, the slow proteolysis (almost inhibition of proteolytic activity) of CA containing glutathione was also observed in some metalloproteases, such as MMP-2 (Bogani et al., 2007). This might imply GSH can inhibit proteases and their catalytic mechanism.

The found protein patterns have also been observed from other research works (Hager et al., 2012b; Torbica et al., 2012; Vallons et al., 2011). The activity of flour endogenous proteases was proven by the presence of identical protein patterns in all fermentations. This can be observed comparing these patterns with the one on CA doughs. Besides, low protein content (lanes with low band intensity) were well linked to the FAN content results. Hence, protein fractions derived from fermentations with high proteolytic activity showed lower band intensity than fractions derived from fermentations with low proteolysis. Proteins analyzed under reducing conditions showed different patterns than proteins analyzed under normal conditions. This means that high molecular aggregates were present and braked down by disulfide bound reduction.

ANOVA showed the significant influence of pH on the activation of endogenous proteases. Indeed, low pH values were responsible for enzymes' activation, which increased proteolysis in SD. However, fermentations with *W. cibaria* (TMW 2.1333) showed another trend due to the high FAN content compared to the one of Mix fermentations, which exhibited lower pH values. Probably, *W. cibaria* (TMW 2.1333) was able to produce compounds which increased proteolysis or it did not consume FAA released by carboxypeptidase activity. In fact, these results show that the pH is not the only parameter capable of influencing proteolysis in buckwheat SD.

Used strains displayed a lower amount of total free amino acids compared to the CA doughs. This behavior might be derived from the activity of carboxypeptidases in CA doughs, while the released FAA were consumed by the employed strains (Dunaevsky and Belozersky, 1989). Indeed, the consumption of several AA, such as lysine, arginine and methionine, was also observed by other authors (Araque et al., 2013; Fernández and Zúñiga, 2006).

4.3 Influence of controlled extracellular ORP in microbial metabolism

In this investigation, the extracellular ORP was held constant obtaining a reducing and oxidizing state of the media. Moreover, the microbial metabolism was also investigated (section 3.3).

These results pointed out the capacity of employed strains to reduce oxygen during control fermentations, as shown previously in section 3.3. Basically, oxygen reduction is linked with NADH oxidase activity but this gene was not found either in *W. cibaria* (TMW 2.1333) or *P. pentosaceus* (2.6) genome (Kim et al., 2011; Midha et al., 2012). Nevertheless, LAB are also able to use other enzymatic activities to reduce oxygen in water, e.g. cytochrome oxidase which is normally activated by heme (Pedersen et al., 2012). Moreover, the respiration is supported by exogenous heme in *W. cibaria*. The employed starter culture showed a similar growth under oxidizing conditions compared to the control conditions, indicating that these strains were able to use oxygen as electron acceptor probably to avoid the oxidative stress during respiration (Brooijmans et al., 2009; Gänzle et al., 2007; Pedersen et al., 2008). However, Mix culture showed a lower growth compared to the other fermentations under reducing condition. Despite the slow growth of the inoculated strains, these fermentations displayed pH and organic acid concentration similar to normal and oxidizing conditions. Hence, the low redox potential might have influenced the enzymatic activity responsible for organic acid production, as described in other research works (Ji-dai et al., 2008; Riondet et al., 2000). *P. pentosaceus* (TMW 2.6) showed an unusual ORP rise under reducing conditions and this phenomenon was observed in fermentations in Spicher medium with the same conditions. This increase might be linked to the production of peroxides to balance the intracellular ORP. Overall, it was demonstrated that the used starter cultures are able to grow in very different extracellular redox potentials. Thus, they are suitable for many different fermentation conditions in the industrial production.

As expected, *W. cibaria* (TMW 2.1333) and Mix culture produced more acetic acid under oxidizing conditions. This effect is well known for heterofermenters, which use oxygen as electron acceptor to synthesize an ATP, as also described by *L. sanfranciscensis* (Stolz et al., 1995). Moreover, as expected, *P. pentosaceus* did not show this behavior because homofermenters cannot synthetize ATP using oxygen as electron acceptor (Gänzle et al., 2007). Basically, acetic acid production is a big issue in the bakery industry due to the yeast inhibition activity of this weak acid during the proofing time (Häggman and Salovaara, 2008). Hence, if heterofermenters are employed in fermentation processes and the decrease

of acetic acid is needed, then it can be possible to remove the oxygen using forming gas. Nevertheless, acetic acid is an important aroma compound for bread, and consequently if the volume is not important for some bakery products, then the fermentation process can be aerated using compressed air to increase the concentration of this metabolite.

The release of inositol in each fermentation is caused by the presence of phytic acid, which is normally degraded by LAB upon fermentation (Haros et al., 2008; Moroni et al., 2012; Palacios et al., 2008). High glucose release under oxidizing conditions might be linked to the use of oxygen as electron acceptor to synthetize a further ATP and probably the use of glucose was unnecessary (Jänsch et al., 2011). On the other hand, the release of glucose might be also come from the synergic effect between extracellular amylase, such as α-amylase, and maltose phosphorylase activity. Indeed, amylases can cleave starch to dextrin and the latter can be hydrolyzed to maltose, which is hydrolyzed into glucose-1-P and glucose. The latter is dismissed outside the cell due to its inappropriate metabolic conversion compared to glucose-1-P (Gänzle et al., 2007; Petrova et al., 2013).

The decrease of thiol content under oxidizing condition in all fermentations could be associated to the oxidation of free thiols in disulfide bonds. Moreover, *P. pentosaceus* (TMW 2.6) showed an increase of thiol content under reducing conditions probably due to the disulfide bonds reduction. Whereas, the decrease of thiols in fermentations with mix culture could be influenced by cysteine consumption.

The observed decrease of FAN and FAA content under oxidizing conditions in fermentation with *W. cibaria* (TMW 2.1333) could depend on the synergic effect between aspartic proteases and the increase of endogenous peptidase activity (Timotijevic et al., 2003). Moreover, this increase under reducing conditions has only been detected with *W. cibaria* (TMW 2.1333), thus reduction does not support proteolysis process. Despite, reducing conditions influenced significantly the FAA release and it could be linked to the low AA consumption of *W. cibaria* (TMW 2.1333) under such conditions.

Extracellular ORP changes showed to have an influence on volatile compounds of buckwheat SD. A major number of volatile compounds has been observed under oxidizing conditions, thus oxidation improves the conversion of amino acids into aroma compounds. However, these results were unexpected because normally the increase of aroma compounds occurs under reducing conditions in wheat system, where extended proteolytic activities influenced the presence of aroma precursors.

4.4 Starter culture screening in buckwheat SD

The goal of this investigation were to screen the microbial metabolism of several LAB using multivariate analysis in buckwheat SD. To monitor the microbial metabolism, different aspects were taken into account: redox potential, proteolysis, carbohydrate consumption, organic acid production, and amino acid consumption. Moreover, the employed LAB were categorized into homofermenters (*homo*), heterofermenters (*hetero*) and facultative heterofermenters (*f. hetero*).

Considering the main metabolism, *hetero* were primarily differentiated over the groups due to the production of ethanol and the higher acetic acid production against lactic acid. Moreover, *hetero* showed a higher thiol content than other groups and it could be associated to the high reduced cofactor's regeneration, which is typical high for heterofermenters (Vermeulen et al., 2006). Furthermore, these results demonstrated the linear correlation between the acetic acid production and the increase of free thiols, as shown by Gänzle et al. (2007), who described a connection of this event with the reducing activity of glutathione dehydrogenase. Instead, *homo* and *f. hetero* presented identical main metabolism, mainly characterized by similar production of organic acids, such as lactic acid. These similarities even derived from low thiol content of some strains within these two groups, such as species of: *L. paralimentarius* (TMW 1.1234 and TMW 1.1235), *L. mindensis* (TMW 1.1206), *P. pentosaceus* (TMW 2.6) and *L. sakei* (TMW 1.22). However, the analogies of the two groups were also influenced by similar FAA content, as showed by *L. paralimentarius* (TMW 1.1235), *L. paracasei* (TMW 1.1305 and TMW 1.1434) and *P. pentosaceus* (TMW 2.1036).

Observing the carbohydrate consumption, the *hetero* displayed a different behavior among the groups due to their consumption of arabinose, and release of mannitol and inositol (Fig. 24b). Basically, the production of mannitol upon LAB fermentation is related to the conversion of fructose, which is used as electron acceptor (Gänzle et al., 2007). However, the carbohydrate consumption of *homo* and *f. hetero* was similar due to their non-consumption of fructose, as observed for by *P. pentosaceus* (TMW 2.6), *L. paracasei* (TMW 1.304), *L. mindensis* (TMW 1.1206), and *L. casei subsp. paracasei* (TMW 1.1462). However, *f. hetero* have been showed some outliers (Fig. 24a) in the second discriminant (LDA2) due to the high content of glucose after fermentation. These strains were: *L. paralimentarius* (TMW 1.1234 and TMW 1.1235), *L. graminis* (TMW 1.1174) and *L. casei subsp. paracasei* (TMW 1.1183).

These results are in agreement with those shown previously in section 3.2. Indeed, here it was demonstrated that flour endogenous proteases mainly influenced proteolysis upon buckwheat SD fermentations. *E. faecalis* TMW 2.630 did not displayed any higher proteolytic activity than other strains, and no statistical significance of FAN content could be detected among the groups. Moreover, in the section 3.2, it has been reported that proteolytic activity was mainly influenced by pH in the range 4.22-3.77 (after 24 h fermentation). Instead, these results showed that pH had no significant influence in the range of 5.38-3.99 after 8 h fermentation. Therefore, it might hypothesized that cysteine proteases were more active in this pH range, considering their optimum of pH 5.5.

Hetero showed a high total AA consumption and it could be explained into two way, either *hetero* converted AA into aroma compounds or they used AA for biomass production. However, it has been observed that the amino acid metabolism was more strain specific due to the presence of many outliers within the groups. Some strains of the three groups (*homo, hetero,* and *f. hetero*) showed the highest total FAA consumption, such as *L. mindensis, L. paracasei*, *L. plantarum,* and *L. brevis*. Moreover, *homo* displayed the high ornithine production but even *W. cibaria* (TMW 2.1333) and some *f. hetero* exhibited this conversion close to the *home*. Normally, LAB convert arginine to ornithine generating ATP and $NH_{3,}$ and through this activity they are able to have energetic advantages and protection from acid stress (Weckx et al., 2010).

These results confirm that AA metabolism is more strain specific and this is also reported in Fig. 25. However, this concept can be also applied to other variables such as carbohydrates.

4.5 Influence of buckwheat SD in GF breads

The goal of this investigation was to observe the effect of different aged (8 and 24 h) oxidizing and reducing SD on buckwheat (BB) and brown rice (BR) bread.

Batters prepared with chemically acidified doughs and sourdoughs showed low electropherogram intensity compared to the control batters. This event is normally caused by protein breakdown during SD fermentations and proofing, where the proteolysis is also influenced by the presence of the yeast (Moroni et al., 2011). However, the proteolytic activity could also be linked to the activation of buckwheat endogenous proteases, as showed in section 3.2. Indeed, the acidification can activate aspartic proteases (Moroni et al., 2011).

BB and BR batters showed very similar protein patterns and it might be linked with the high protein concentration of buckwheat flour (even present in BR recipe) which hides the low concentration of brown rice protein fraction (Hager et al. 2012b). Moreover, BR batters exhibited a higher protein content, which might be derived from an increase of solubility compared to the BB batters, which had a higher pH. Unfortunately, no evident effects were recognized on protein patterns through the addition of oxidized (with *Pediococcus pentosaceus* TMW 2.6) and reduced (with *Enterococcus faecalis* TMW 2.630) SD. Indeed, band intensity's changes were expected due to reduction or oxidation but this event did not appear. Thus, redox agents did not affect the protein structure of GF proteins. The reasons could be many, e.g., disulfide bonds were not well solubilized and therefore redox agents could not react with them. Otherwise, the concentration of molecular oxygen present in the dough was not high enough to oxidize free thiols into disulfide bonds, as described in wheat dough (Joye et al., 2009). Hence, since the redox agents cannot affect the protein network, the use of oxidizing and reducing SD was not able to influence differently the microstructure of BB and BR after proofing time. Overall, both proteolysis and redox agents were not capable of influencing the interaction between proteins and flour components. Moreover, it was observed that the higher interaction activity of BR batters could not influence positively the protein structure in comparison with BB batters. In fact, this event might be linked to the protein-starch interactions among the employed flours, i.e., brown rice flour showed low stable interactions. Low protein contents intensified the elasticity of BB batters and this effect was also described in works where buckwheat was treated with commercial proteases (Renzetti and Arendt, 2009b). Moreover, close values of the phase angle among several trials have also been detected in brown rice batters treated with commercial proteases (Renzetti and Arendt, 2009a). However, the lacking changes of dough microstructure among different

samples might depend on the temperature of the measurements (30 °C). This phenomenon has also been described by Clarke et al., (2002), indeed they did not find any relevant differences of dough rheology among different trials containing different sourdoughs. It might be hypothesized that the interactions of dough components (mainly starch and proteins) are not detectable at 30 °C and it is partially confirmed by the results of BB temperature sweeps displayed on Fig. 27a. In fact, doughs containing pre-doughs with low protein content (high proteolysis after long fermentation times) displayed high gelatinization peaks between 75 and 80 °C, while BB control doughs (C and C-GSH) showed lower peaks. Moreover, this effect was also observed in other research works (Moore et al., 2007; Moroni et al., 2011b; Zheng et al., 1998). Indeed, low concentrations of proteins can improve the gelatinization capacity of starches, which have more freedom for the water uptake. However, this improvement has not been detected in BR batters and this could mean that there was a protein concentration which kept constant the gelatinization activity of the starch (Renzetti et al., 2010).

Compared to the controls (including CA), an increase of bread volume could be detected in BB breads baked with SD. Thus, the proteolysis could be responsible for this effect, which is normally associated to the starch pasting temperature or to the batter extendibility (Kusunose et al., 1999; Renzetti and Arendt, 2009b). However, batters containing SD showed very similar pasting properties compared to the CA batters, indicating that volume increase might derive from the positive activity of employed strains on yeast gas production. Probably, this strains did not produce compounds, which are responsible for yeast inhibition during the proofing time, such as acetic acid (Gobbetti et al., 1995).

A correlation was observed between volume and hardness, which is influenced by the presence of big cells, which give soft bread slices. Therefore, this correlation can explain the softness of BB sourdough breads. Moroni et al., (2011) showed buckwheat breads (prepared with SD) with low volume. In fact, the authors used a starter culture that probably inhibited the yeast activity during the proofing time. Moreover, BR sourdough breads displayed big voids compared to the control breads and even here the employed strains probably influenced positively the yeast activity. Normally, big cavities can caused by coalescence due to hardening of cell wall (Hayman et al., 1998). Moreover, BR breads have been showed a high crumb hardness compared to BB breads and it depends on the low volume and high cell density of BR breads due to the correlation among these variables. Furthermore, bread characteristics showed no differences among the SD breads, indicating the lacking effect of redox agents. Indeed, this is also confirmed by the values of breads prepared with 3 mM

GSH, which are smaller than BB sourdough breads. If redox agents would have any effects on the bread, then we would probably obtain the same performance between reducing SD bread and bread containing GSH. The use of chemically acidified doughs and SD have been showed a volume decreasing trend, which could be linked to the higher concentration of acetic acid contained in 8 h SD or CA. Indeed, the application SD containing different concentration of acetic acid can influence differently the gas production and thus the final bread volume (Gobbetti, 1998). It has been demonstrated that heterofermenters, such as *L. sanfranciscensis*, negatively affects the yeast activity during the proofing time due to the high production of acetic acid compared to homofermenters and facultative heterofermenters (Gobbetti et al., 1995).

Overall, this investigation demonstrated the non-influence of oxidizing and reducing SD on GF breads. However, it should be consider the different effects, which different bread making conditions can have on the final bread properties, as even shown by other authors (Demirkesen et al., 2011; Gómez et al., 2012). Nonetheless, the effect of higher concentrations of redox reagents in GF breads remains unclear, as also shown by other authors (Yano, 2012, 2010; Yano et al., 2013).

4.6 Effect of LAB-yeast interaction on GF bread volume

More baking tests were performed using three different dry yeast and five different sourdoughs. On average, bread prepared with CA doughs showed a non-significant volume increase compared to control breads. This could probably depend on the high content of carbohydrates, which might be used from the yeasts during the proofing time. However, free carbohydrates were detected even in the employed SD but they showed a higher bread volume than CA and Fresh breads. These results demonstrated the improvement of the leavening capacity using SD. Probably, the employed starter culture did not produce enough acetic acid, which decreases the activity of *Saccharomyces cerevisiae* (Gobbetti, 1998). Moreover, yeast-LAB interactions could also be influenced by substrate consumption (Gobbetti, 1998). Indeed, most of the yeasts are able to consume hexoses, such as maltose. Furthermore, to avoid the competition with the yeast, LAB should grow on different substrate than yeast and vice versa. Nevertheless, the maltose consumption of *L. sanfranciscensis* releases excessed glucose, which can be used by the bakery yeast (Gobbetti, 1998). Moreover, employed starter culture were able to hydrolyze starch granules as well as flour endogenous enzymes. Thus, employed strains did not mainly compete with employed yeasts for substrate consumption, except for LAB1 and LAB5 combined with rp yeast. Otherwise, SD breads would had showed lower volumes than CA breads.

Low pH values of the doughs influenced negatively the volume of breads prepared with Pante red yeast. This could depend on the low intolerance of some *Saccharomyces cerevisiae* strains in acidic environments. Thus, breads prepared with LAB2 and LAB5 showed a lower volume compared to the other SD breads. However, the pH range (5.7-5.2) of employed batters was not big enough to decrease extremely the gas production during the proofing. Moreover, the stress tolerance of yeast is not only strain dependent but it is also species dependent. Indeed, *Candida milleri* is more tolerant to acetic acid than *Saccharomyces cerevisiae* (Gobbetti, 1998; Häggman and Salovaara, 2008). Considering AA metabolism, there is no a really competition between yeasts and LAB, because a continuous exchange of AA occurs during SD fermentation. Indeed, *S. cerevisiae* releases alanine, isoleucine, glycine, valine, and proline, while LAB release mainly glycine and alanine (Collar, 1996).

The lack of clear correlations between bread characteristics and batter rheology confirms the positive influence of SD on yeast gas production during the proofing time. Thus, these results prove the gas improvement by LAB during the proofing time as also showed in section 3.5. Furthermore, this outcome is even in agreement with the results of buckwheat dough's

protein fraction, reported in section 3.5. Indeed, if modifications of the dough's protein fraction would have occurred, then linear correlations between bread properties and rheology would be more evident. However, LAB or other compounds could be also able to inhibit yeast gas production, e.g. high amounts of acetic acid inhibit the yeast activity. Moreover, other than weak acids, furans and phenolic compounds can also inhibit yeasts growth and metabolic activity (Almeida et al., 2007). Indeed, Buckwheat flour contains phenolic compounds and furans (Pomeranz, 1983; Prosen et al., 2010). Nonetheless, LAB can help to reduce the content of phenols during fermentations due to the capacity to produce specific enzymes which are able to degrade this yeast's inhibitors (Curiel et al., 2010; Rodríguez et al., 2009). Thus, this could explain the positive effect of the addition of SD to the volume increase of buckwheat breads.

5 Summary

Celiac disease is an immune-mediated disease, which interests almost 1% of the populations in the western world, as even mentioned in the introduction. The only solution for this people, to have a healthy nutrition and avoid problems, is to conduct a gluten-free diet. Thus, these type of patients should not ingest foods containing gluten residuals. Foods are accounted as GF if they are prepared with ingredients, which do not contain prolamins from *Triticum* species and the level of gluten must not exceed 20 mg/Kg. Moreover, it can also be considered GF foods containing ingredients deriving from *Triticum* species, which were reconverted in GF and the level of gluten must not exceed 200 mg/Kg (Arendt et al., 2008a). Unfortunately, the absence of gluten is a very big issue especially in bakery product, since they show a low quality compared to the gluten-containing foods. Thus, it is a huge challenge for food technologist to produce GF bakery products. To date, several gluten-substituting ingredients and additives were employed to improve the quality of these products. However, these applications were partially successful due to their high price and dietary intolerance. Thus, to avoid these issues sourdoughs are normally used in GF formulations to improve the aroma and the structure properties of GF breads.

Nowadays, it is still unknown the effect of microbial activity on redox potential and proteolysis of GF flours. Thus, the goals of this work were the following:

- investigation of reducing activity of LAB in buckwheat sourdoughs;
- investigation of synergic effect between reduction and proteolysis in buckwheat SD;
- investigation of the effect of 'redox' SD on buckwheat protein fraction;
- investigation of the effect of 'reducing' or 'oxidizing' buckwheat SD on the properties of GF breads.

In this work, it was shown the reducing activity of LAB using redox potential measurements. According to the actually literature, this type of measurements were used for the first time ever in sourdough fermentations. The reproducibility of ORP measurements was demonstrated. Moreover, LAB reduced the SD in the same manner as in other food matrix. Moreover, *P. pentosaceus* (TMW 2.6) exhibited a low reduction, almost oxidizing compared to the other employed strains. The reduction step occurred simultaneously with the oxygen reduction during SD fermentations. Furthermore, the highest metabolites production rate was reached after the reducing step in all fermentations. LAB showed reducing activity, and each strain exhibited different ORP time courses; therefore, redox potential measurements

displayed capability for further LAB microbial screening. Besides, these measurements might be applied for detection of microbial contaminations during SD fermentations.

It was shown that proteolytic activity was mainly influenced by buckwheat flour endogenous proteases. Indeed, chemically acidified doughs (non-inoculated doughs) showed a higher proteolysis as well as buckwheat sourdoughs inoculated with starter culture. Moreover, the employed LAB, except the control proteolytic strain *E. faecalis* (TMW 2.630), did not display any extracellular proteolytic activity. However, the proteolysis process was influenced by pH and not by ORP changes, i.e., acidification and thus low pH values activated buckwheat flour endogenous proteases and probably aspartic proteases are more active at low pH. ORP changes were correlated with free thiol content changes, i.e., low ORP values were linked to high free thiol content. Moreover, the addition of redox agents, such as glutathione, showed an inhibition of proteolysis during the fermentations time, indicating the possible inhibition of some metalloproteinases. Furthermore, proteolysis showed a clear effect on the band intensity of protein patterns in SD and CA doughs. However, reducing or oxidizing SD did not display any detectable effect on the electrophoretic patterns of protein fractions, e.g., fractions of fermentations with *P. pentosaceus* (TMW 2.6) and *E. faecalis* (TMW 2.630).

Changes of extracellular ORP influenced LAB microbial metabolism, e.g., *W. cibaria* (TMW 2.1333) and Mix culture produced more acetic acid under oxidizing conditions. Different extracellular ORP was not relevant on the growth (biomass production) of *P. pentosaceus* (TMW 2.6), *W. cibaria* (TMW 2.1333) and mix culture, demonstrating the ability of these strains to grow under extremely different redox potentials. Moreover, oxidizing conditions decreased the free thiol content of the buckwheat SDS-soluble fraction. Furthermore, oxidizing states increased the number of volatile compounds in buckwheat SD fermentations. However, even these results confirmed that reducing ORP did not increase proteolysis but the employed strains consumed less FAA under those conditions. Thus, FAA release was detected with low ORP values (reducing conditions).

Even the results of microbial screening confirmed that proteolysis is mainly caused by buckwheat flour endogenous proteases. Indeed, no significant differences of FAN content were shown among the microbial groups. Moreover, the pH did not influence significantly the proteolysis in the range of 5.38-3.99, indicating the probably homogenous activity of cysteine protease. Furthermore, fermentations with *hetero* displayed the highest content of free thiols of buckwheat SDS-soluble fraction. *Hetero* were also mainly discriminated from

homo and *f. hetero* due to the higher production of acetic acid and mannitol. On the other hand, *homo* showed a similar AA metabolism like *f. hetero* but they differentiated a bit from the latter due to the high ornithine production. Indeed, a big part of *f. hetero* were not able to use arginine and thus to convert it in ornithine. However, statistical analysis showed the presence of outliers among the groups, indicating that several microbial activities were more strain-specific than group-specific.

Baking tests displayed an increase of viscoelasticity of BB doughs prepared with CA and SD. Moreover, extended proteolysis of buckwheat SD showed an improvement of starch gelatinization capacity. However, rheological measurements did not show relevant differences of batters containing reducing (*E. faecalis* TMW 2.630) and oxidizing (*P. pentosaceus* TMW 2.6) SD. The same result occurred in protein analysis of batters' fraction. Indeed, no differences were detected between patterns of reducing or oxidizing doughs. In fact, only a common proteolysis occurred in each samples after proofing time. BB breads prepared with SD showed higher volume and lower softness than control breads, indicating the positive influence of SD on the yeast activity during proofing time. BR breads containing SD did not display relevant differences compared to controls except that they exhibited big voids in the crumb structure.

Moreover, in section 3.6, the results of section 3.5 were confirmed, i.e., the employed SD displayed a positive effect on the gas production during the proofing time of GF breads.

In conclusion, these results demonstrated the main role of flour endogenous proteases and the correlation between ORP and content of free thiols in buckwheat sourdoughs. Moreover, proteolysis was more influenced by acidification, while it was not affected by microbial reduction of addition of redox agents, such as glutathione. Unfortunately, redox agents or 'reducing' and 'oxidizing' starter culture did not affect the protein faction of either SD or batters. Thus, even breads prepared with different SD did not show any relevant differences in baking performances. However, something interesting was observed. Indeed, SD showed to have an influence on the yeast activity during the proofing time, thus on the final bread volume and crumb hardness. Hence, these results clearly indicated that the application of appropriate sourdoughs can improve the baking performances of GF breads.

6 Zusammenfassung

Zöliakie ist eine Krankheit, die 1% der westlichen Bevölkerung betrifft. Die einzige Lösung zur Vermeidung gesundheitlicher Probleme ist eine lebenslange glutenfreie Diät. Lebensmittel können als glutenfrei deklariert werden, wenn sie mit Zutaten zubereitet werden, die keine Prolamine aus *Triticum* species und nicht mehr als 20 mg/kg Gluten enthalten. Darüber hinaus kann man auch Lebensmittel als glutenfrei bezeichnen, die Zutaten aus *Triticum* species enthalten, welche in glutenfrei umgewandelt wurden und deren Glutengehalt nicht mehr als 200 mg/kg aufweist. Leider ist die Abwesenheit von Gluten in Backwaren ein großes Problem, da sie im Vergleich zu glutenhaltigen Lebensmitteln eine niedrige Qualität aufweisen. Deshalb ist es für Lebensmitteltechnologen eine große Herausforderung, glutenfreie Backwaren weiterzuentwickeln. Bisher wurden zahlreiche Zutaten und Zusatzstoffe eingesetzt, um Gluten zu ersetzen und so die Qualität dieser Produkte zu verbessern. Aufgrund ihres hohen Preises und der Lebensmittelintoleranz waren diese Anwendungen jedoch nur teilweise erfolgreich. Um diese Probleme zu vermeiden, werden in glutenfreien Rezepturen normalerweise Sauerteige verwendet, die die Aroma- und Struktureigenschaften von glutenfreiem Brot verbessern können.

Bisher ist der Effekt von mikrobieller Aktivität auf das Redoxpotential und die Proteolyse in glutenfreiem Mehl unbekannt. In dieser Arbeit sollten deswegen die Reduktionsaktivität von Milchsäurebakterien in Buchweizensauerteigen und Synergieeffekte zwischen Reduktion und Proteolyse im Buchweizensauerteig untersucht werden. Darüber hinaus sollte der Effekt reduzierter bzw. oxidierter Sauerteige auf Buchweizen-Proteinfraktionen sowie auf die Eigenschaften von glutenfreien Broten erforscht werden.

In dieser Arbeit wurde die reduzierende Aktivität von Milchsäurebakterien mittels Redoxpotential-Messungen beobachtet. Gemäß der aktuellen Literatur, wurde diese Art der Messung das erste Mal bei Sauerteigfermentationen angewandt. Es wurde die Reproduzierbarkeit von ORP Messungen sowie die Tatsache, dass Milchsäurebakterien den Sauerteig in derselben Art und Weise wie in anderen Nahrungs-Matrizen reduzieren. *P. pentosaceus* (TMW 2.6) wies eine geringe Reduktion, im Vergleich mit den anderen Stämmen einen fast oxidierenderen Effekt auf. Die Reduktionsphase trat gleichzeitig mit der Sauerstoffreduktion während der Sauerteigfermentationen auf. Darüber hinaus wurde die höchste Reduktionsrate von Metaboliten nach Reduktionsphasen in allen Fermentationen erreicht. Obwohl LAB eine reduzierende Aktivität aufweisen, zeigt jeder Stamm unterschiedliche ORP-Verläufe. Deshalb stellen Reduktionpotentialmessungen ein Potential

für weitere LAB mikrobieller Screenings dar. Deswegen können diese Messungen für den Nachweis von mikrobieller Kontamination während Sauerteigfermentationen verwendet werden.

Diese Ergebnisse haben gezeigt, dass die proteolytischen Aktivität hauptsächlich durch buchweizenendogene Proteasen beeinflusst wird. In der Tat weisen chemisch gesäuerte Teige (nicht-angeimpfte Teige) eine höhere Proteolyse auf wie auch in Buchweizensauerteigen, die mit Starterkulturen angeimpft wurden. Außer dem proteolytischen Stamm *E. faecalis* (TMW 2.630), wiesen alle eingesetzten LAB keine extrazelluläre proteolytische Aktivität auf. Allerdings wurde die Proteolyse hauptsächlich von pH und nicht von ORP beeinflusst, d.h. niedrige pH-Werte aktivierten endogene Proteasen von Buchweizenmehl bzw. aspartische Proteasen, die normalerweise unter niedrigen pH-Werte aktiver sind. Darüber hinaus stellte die Zugabe von Redoxreagenzien, bzw. Glutathion, eine Inhibition der Proteolyse während der Fermentationszeit dar. Durch die Proteolyse wurde ein deutlicher Effekt auf die Bandenintensität der Proteinmuster von SD und CA Teige erkennbar. Allerdings zeigten entweder reduzierende oder oxidierende SD keine wesentliche Wirkung auf elektrophoretische Muster bzw. Proteinfraktionen von Fermentationen mit *P. pentosaceus* (TMW 2.6) und *E. faecalis* (TMW 2.630).

Veränderungen an extrazelluläre ORP beeinflussten LAB-Stoffwechsel, so dass z.B. unter oxidierende Bedingungen bei *W. cibaria* (TMW 2.1333) und Mix-Kultur mehr Essigsäure gebildet wurde. Unterschiedliche ORP-Werte haben keine relevanten Einfluss auf das Wachstum (Biomasse) von *P. pentosaceus* (TMW 2.6), *W. cibaria* (TMW 2.1333) und Mix-Kultur. Außerdem sank der freie Thiolgehalt der SDS-löslichen Buchweizenfraktion von oxidierende Bedingungen. Darüber hinaus wurden flüchtige Metaboliten während der SD Fermentationen durch oxidierende Bedingungen erhöht. Allerdings haben diese Ergebnisse auch bestätigt, dass reduzierende Bedingungen die Proteolyse nicht verbessert haben.

Die Resultate der mikrobiellen Screenings haben sogar bestätigt, dass die endogenen Proteasen von Buchweizen auf die proteolytische Aktivität eine entscheidende Rolle spielen. Tatsächlich wurden keine signifikanten Unterschiede des FAN-Inhaltes unter den unterschiedlichen mikrobiellen Gruppen festgestellt. Außerdem hat der pH-Wert keine signifikanten Unterschiede auf die Proteolyse zwischen pH 5.35-3.99 dargestellt und das bedeutet, dass Cysteinproteasen wahrscheinlich in dem pH-Bereich aktiver waren. Zudem wiesen Fermentationen mit Heterofermenter eine höhere Konzentration an freien Thiolgruppen der SDS-lösliche Fraktion auf. Heterofermenter unterschieden sich

hauptsächlich von den anderen Gruppen aufgrund der höheren Bildung von Essigsäure und Mannitol. Anderseits weist Heterofermenter einen ähnlichen Aminosäurestoffwechsel wie die fakultativen Hetrofermenter auf, wobei der Hauptunterschied zwischen den beiden die Ornithinbildung war (Heterofermenter bildeten Ornithin aus Arginin). In der Tat war ein großer Teil der *f. hetero* nicht in der Lage Arginin zu verwenden und es letztlich in Ornithin umzuwandeln. Allerdings haben statistische Analysen das Vorhandensein von Ausreißern innerhalb der Gruppen bewiesen. Daher zeigt dieses Phänomen, dass die mikrobiellen Aktivitäten teilweise mehr stammspezifisch als stoffwechselspezifisch sind.

Während der Backversuche konnte man feststellen, dass die Viskoelastizität der BB Teige, welche mit CA und SD hergestellt wurden, zugenommen hat. Darüber hinaus zeigt eine erweiterte Proteolyse von Buchweizensauerteige eine Verbesserung der Gelbildung der Stärke. Allerdings zeigten rheologische Messungen keine relevanten Unterschieden zwischen Teige mit reduzierenden oder oxidierenden Sauerteigen. Das gleiche Ergebnis kommt bei der Proteinanalyse vor, in der eine einheitliche Proteolyse aufgewiesen wird. Die mit SD hergestellte BB Brote zeigten im Vergleich mit den Kontrollbroten ein höheres Volumen und eine niedrigere Weichheit, d.h. die Zugabe von Sauerteig hatte während der Gare einen positiven Einfluss auf die Hefeaktivität. Anderseits hat die Zugabe von Sauerteig, außer der Bildung großer Löcher in der Krumenstruktur, keinen relevanten Unterschied in BR Broten. In Kapitel 3.6 wurden diese Ergebnisse zudem bestätigt, d.h. die verwendeten Sauerteige haben einen Einfluss auf die Gasbildung während der Gare von glutenfreien Teige.

Fazit, die Ergebnisse dieser Dissertation zeigen die Hauptrolle der endogenen Proteasen sowie den Zusammenhang zwischen ORP und Inhalt an freien Thiolgruppen in Buchweizensauerteigen. Außerdem wurde die Proteolyse eindeutig von der Säuerung und nicht von Redoxreagenzien wie Glutathion beeinflusst. Leider wurden Proteinfraktionen von Sauerteigen und Teigen nicht von Redoxreagenzien oder reduzierende und oxidierende Starterkulturen beeinflusst. Deshalb zeigten Broten mit unterschiedlichen Sauerteigen keine relevanten Unterschiede der Backeigenschaften auf. Allerdings wurde etwas Interessantes herausgefunden, und zwar, dass die Sauerteige einen positiven Effekt auf die Gasbildung der Hefen haben, was letztendlich einen Effekt auf das Brotvolumen und Krumenweichheit hat. Deshalb weisen diese Ergebnisse darauf hin, dass die Anwendung eines richtigen Sauerteiges in glutenfreien Backawaren von großer Bedeutung ist.

7 Appendix

7.1 Vorveröffentlichungen (list of publications which resulted from this dissertation)

Papers:

- Capuani, A., Behr, J., Vogel, R.F., 2012. Influence of lactic acid bacteria on the oxidation– reduction potential of buckwheat (*Fagopyrum esculentum Moench*) sourdoughs. European Food Research and Technology. DOI: 10.1007/s00217-012-1834-4. (Capuani et al., 2012)
- Capuani, A., Behr, J., Vogel, R.F., 2012. Influence of lactic acid bacteria on redox status and on proteolytic activity of buckwheat (Fagopyrum esculentum Moench) sourdoughs. International Journal of Food Microbiology. DOI: 10.1016/j.ijfoodmicro.2013.04.020. (Capuani et al., 2013a)
- Capuani, A., Werner, S., Behr, J., Vogel, R.F., 2012. Effect of controlled extracellular oxidation–reduction potential on microbial metabolism and proteolysis in buckwheat sourdough. European Food Research and Technology. DOI: 10.1007/s00217-013-2120-9. (Capuani et al., 2013b)
- Capuani, A., Behr, J., Arendt, E.K., Vogel, R.F., 2014. Impact of "oxidizing " and " reducing" buckwheat sourdoughs on brown rice and buckwheat batter and bread. Eur. Food Res. Technol. DOI: 10.1007/s00217-014-2175-2 (Capuani et al., 2014a)
- Elektrochemische Prozessanalytik: Redoxpotentialmessungen als Kontrollparameter in Sauerteigfermentationen. GIT Laborfachzeitschrift 58. Jahrgang, 2014
- Capuani, A., Stetina, M., Gstattenbauer, A., Behr, J., Vogel, R.F., 2014. Multivariate analysis of buckwheat sourdough fermentations for metabolic screening of starter cultures. Int. J. Food Microbiol. 185, 158–166. (Capuani et al., 2014b)

Submitted:

- Impact of sourdough on baking performances of commercial dry yeasts
- Oxidation-reduction potential measurements as online monitoring tool in sourdough fermentations
- Redoxpotentialmessungen als Online-Monitoring-Tool in Sauerteigfermentationen

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