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SOURDOUGH BIOTECHNOLOGY FOR IMPROVING BREAD NUTRITIONAL PROPERTIES

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Esta Tese foi julgada adequada para obtenção do Título de Doutora em Ciência dos Alimentos e aprovada em sua forma final pelo Programa ...

Local, 15 de março de 2019.

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À minha nona Santina Giusti Acordi.
(in memorian)
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RESUMO

A fermentação *sourdough* tem sido estudada por sua capacidade de melhorar as propriedades sensoriais, nutricionais e tecnológicas de pães, resultado do metabolismo da comunidade microbiana do *sourdough*, composta principalmente por bactérias ácido-láticas (BAL) e leveduras. O objetivo deste estudo foi caracterizar a evolução da comunidade microbiana de *sourdoughs* produzidos no Brasil durante as etapas de preparo (propagação) e investigar os efeitos da fermentação *sourdough* na concentração de FODMAPs (do inglês, *Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols*) em pães. FODMAPs são uma classe de carboidratos associados ao desencadeamento dos sintomas da Síndrome do Intestino Irritável (SII) e da Sensibilidade Não Celíaca ao Glúten (SNCG). Duas temperaturas foram testadas para propagação, 21 ºC ± 1ºC (SD1) e 30 ºC ± 1ºC (SD2). A composição microbiana dos *sourdoughs* foi investigada por métodos dependentes e independentes de cultivo. A concentração de FODMAPs foi avaliada por métodos enzimáticos e por Cromatografia Líquida acoplada à Espectrometria de Massas (LC/MS-MS). A comunidade microbiana da farinha de trigo foi composta exclusivamente por *Proteobacteria* (*Escherichia hermannii, Kosakonia cowanii, Pantoea ananatis* e algumas espécies de *Pseudomonas*). BAL foram detectados somente após a primeira etapa de propagação (BS1) e tornaram-se predominantes (*Lactobacillus farciminis* e *Lactobacillus curvatus*) após a quarta etapa (BS4). A partir desta etapa, a diferença entre as temperaturas afetou a dinâmica microbiana entre os dois tratamentos. Ao final, a comunidade microbiana do SD1 foi composta exclusivamente por BAL (*L. farciminis, L. curvatus, Leuconostoc citreum* e *Pediococcus pentosaceus)*. *Bacillus, Pseudomonas, Enterococcus* e *enterobacteria* foram inibidos gradualmente durante a propagação e não foram detectados na etapa final. Por outro lado, a temperatura de 30 ºC ± 1ºC favoreceu a presença de bactérias atípicas (que não BAL) para o SD2. *L. farciminis* e *Escherichia hermannii* foram dominantes. *Clostridium*, *Bacillus*, *Enterococcus* foram inibidos, no entanto, *Kosakonia cowanii*, *Pantoea ananatis* e algumas espécies de *Pseudomonas* ainda estavam presentes ao final da propagação. Em relação aos FODMAPs, sacarose, frutose e glicose foram completamente consumidas a partir da primeira etapa de fermentação, enquanto os demais subgrupos dos FODMAPs tiveram suas concentrações reduzidas após a terceira e quarta etapas de fermentação, coincidindo com o predomínio da BAL na comunidade microbiana. Os

pães fermentados por *sourdough* apresentaram maiores proporções de ácidos orgânicos e menor concentração de sacarose, frutose, glicose e frutanos comparados aos pães fermentados por *Saccharomyces cerevisiae*. A concentração de frutanos, o subgrupo mais importante de FODMAPs, foi de 40 a 62% menor nos pães *sourdough*. Portanto, a fermentação *sourdough* representa uma alternativa promissora para o desenvolvimento de pães que possam ser consumidos por indivíduos que possuam SSI e/ou SNCG.

Palavras-chave: *Sourdough*. FODMAPs. Frutanos. Bactérias Ácido-láticas. Temperatura. Dinâmica Microbiana.

RESUMO EXPANDIDO

Introdução

Alimentos derivados de cereais são componentes importantes da dieta, dentre os quais, destaca-se o pão, um dos principais alimentos consumidos diariamente em todas as partes do mundo. A fermentação de pães tem sido praticada ao longo de milênios, por meio do *sourdough*, uma mistura de farinha e água, fermentada por bactérias ácido-láticas (BAL) e leveduras. Inúmeros estudos têm confirmado a capacidade da fermentação *sourdough* para promover modificações benéficas nas propriedades nutricionais, tecnológicas e sensoriais de pães. A contribuição do *sourdough* na qualidade do pão é decorrente das conversões microbianas e enzimáticas ocorridas durante o preparo da massa e dependente do metabolismo da comunidade microbiana presente no processo de fermentação. Mais de 80 espécies de bactérias já foram identificadas em *sourdoughs* em diversas partes do mundo. No entanto, um estudo deste caráter ainda não havia sido realizado no Brasil. A atividade metabólica da comunidade microbiana afeta macro e micronutrientes dos pães, dentre as quais, a fermentação dos carboidratos é considerada a modificação mais expressiva. O termo FODMAPs (do inglês, *Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols*) é utilizado para descrever carboidratos fermentáveis de cadeia curta, um grupo heterogêneo de moléculas mal absorvidas durante o processo digestivo, com um amplo efeito sobre os processos gastrointestinais, incluindo-se o desencadeamento dos sintomas da Síndrome do Intestino Irritável (SII). Além disso, a ingestão de FODMAPs tem sido recentemente associada ao desencadeamento dos sintomas da Sensibilidade ao Glúten Não Celíaca (SGNC). As manifestações clínicas para ambas as condições são similares e incluem distensão e dor abdominal, diarreia, flatulência e mudança no padrão evacuatório, fadiga e depressão. Enquanto a SII acomete de 7 a 21% da população global, a SGNC afeta cerca de 0,6 a 6%, sendo frequente que um paciente apresente as duas condições simultaneamente. Apesar da conversão de hexoses e pentoses em ácidos orgânicos, inerente ao processo fermentativo, estar bem documentada na literatura, o metabolismo de FODMAPs por BAL e leveduras constitui um campo de pesquisa recente. Produtos derivados de trigo constituem a maior fração de FODMAPs ingeridos na dieta diária, portanto, conhecer a potencialidade da fermentação *sourdough* em reduzir a concentração desses compostos em pães é de interesse clínico, científico e industrial.

Objetivos

O objetivo deste trabalho foi avaliar a diversidade microbiana de *sourdoughs* produzidos no Brasil e estudar a influência do *sourdough* no conteúdo de FODMAPs em pães elaborados por fermentação natural. Os objetivos específicos foram definir dois protocolos de produção de *sourdough* e verificar a influência da temperatura na dinâmica microbiana durante as etapas de propagação; enumerar, isolar e identificar geneticamente BAL, presentes nas etapas de propagação dos *sourdoughs*, aplicando técnicas moleculares de cultivo dependente e independente; desenvolver método para identificação e quantificação de FODMAPs e ácidos orgânicos por *High Performance Liquid Chromatography* (HPLC) e *Liquid Chromatography Tandem-Mass Spectrometry* (LCMS/MS); acompanhar a evolução da microflora durante a elaboração dos *sourdoughs* e correlacioná-la ao metabolismo de FODMAPs e ácidos orgânicos; aplicar os *sourdoughs* na elaboração de pães e verificar a potencialidade da fermentação *sourdough* para a obtenção de pães reduzidos em FODMAPs.

Metodologia

Os *sourdoughs* foram preparados seguindo-se o protocolo tradicional (*sourdough* Tipo I), no qual uma parte da massa pré-fermentada é utilizada para fermentações subsequentes. Farinha de trigo orgânica e água foram misturadas e fermentadas espontaneamente por 48 horas, a 24ºC. A mistura fermentada foi utilizada como inóculo para as subsequentes etapas de propagação (*backslopping steps* – BS), nas quais uma parte da massa pré-fermentada era adicionada de água e farinha de trigo e novamente fermentada a 21 ºC ± 1ºC (*sourdough* 1 – SD1) ou a 30 ºC ± 1ºC (*sourdough* 2 – SD2). Nove etapas de propagação (BS1 – BS9) foram realizadas até que o pH e a acidez titulável estivessem relativamente constantes. Em seguida, os *sourdoughs* foram aplicados na elaboração de pães. Farinha de trigo, água e sal foram misturados, adicionados de *sourdough* e fermentados por 12 horas. A caracterização da microflora dos *sourdoughs* foi realizada por enumeração e isolamento de colônias em diferentes meios de cultura, por sequenciamento do tipo *paired-end* MiSeq Illumina, *Length Heterogeneity-PCR* (LH-PCR), *Repetitive Element Palindromic-PCR* (REP-PCR) e sequenciamento do gene 16S rRNA, em todas as etapas de *backslopping*. A concentração de ácidos orgânicos e FODMAPs foi determinada durante as etapas de propagação dos *sourdoughs* e nos pães por HPLC e LCMS/MS.

Resultados e Discussão

A contagem de bactérias presumivelmente ácido-láticas em mMRS foi de 3 log UFC.g^{-1} para a farinha de trigo. Após a primeira etapa de propagação do fermento (BS2), a densidade celular aumentou para 5,7 log UFC.g^{-1}. A partir do BS5, a densidade celular atingiu 7,5 log CFU.g^{-1} para ambos os *sourdoughs*, apresentando pequenas flutuações até o BS9. Apenas uma pequena parte da população microbiana de ambas as amostras pôde ser recuperada pelo método dependente de cultivo. Dentre os isolados, 11 biotipos pertencentes às espécies *Lactobacillus farciminis, Lactobacillus brevis*, *Lactococcus lactis, Leuconostoc citreum* (02 biotipos), *Enterobacter hormaechei*/*cloacae, Enterococcus gilvus, Eenterococcus hirae, Enterecoccus durans, Enterococcus faecium* e *Enterococcus faecalis*. Através do sequenciamento Illumina MiSeq, espécies de BAL não foram detectadas na farinha de trigo. *Escherichia hermannii, Kosakonia cowanii, Pantoea ananatis* e *Pseudomonas rhodesiae* foram encontradas predominantemente, todas pertencentes ao filo *Proteobacteria*. Após o BS1, o filo *Firmicutes* tornou-se predominante – espécies pertencentes ao gênero *Clostridium* e ao grupo *Bacillus cereus*. *Lactobacillus curvatus, Lactococcus lactis, Leuconostoc citreum* e *Pediococcus pentosaceus* foram detectados pela primeira vez. Após a segunda etapa de fermentação (BS2), a população de BAL superou o gênero *Clostridium*. O fermento foi dominado por *L. curvatus, Clostridium saccharobutylicum* e *L. farciminis. L. graminis, Lactobacillus kimchiensis, Lactobacillus plantarum and Lactobacillus sakei* foram detectados pela primeira vez. Na quarta etapa de propagação (BS4), *L. curvatus* e *L. farciminis* tornaram-se predominantes. A partir desta etapa, a diferença de temperatura afetou a dinâmica microbiana entre os dois *sourdoughs*. Para o SD1, na 5ª etapa de propagação (BS5), *L. curvatus* foi superado por *L. farciminis*. *B. cereus, Enterococcus* e *Enterobacter* foram inibidos. No BS7 e BS9, o fermento a microbiota do fermento foi composta por *L. farciminis*. *L. curvatus, Ln. citreum* e *Pd. pentosaceus* compuseram a população subdominante. *Bacillus, Pseudomonas, Enterococcus* e enterobacteria não foram detectados. Para o SD2, além de *L. farciminis* e *L. curvatus, E. hermannii* e *L. brevis* foram detectados predominantemente. No BS7, *L. farciminis* manteve-se predominante, porém, a população de *L. curvatus* foi reduzida, enquanto as de *E. hermannii* e *K. cowanii* aumentaram. Por fim, no BS9, a comunidade microbiana do SD2 foi composta por *L. farciminis* e *E. hermannii*, predominantemente. *Bacillus*, *Enterococcus* e enterobacteria não foram detectados, no

entanto, *K. cowanii*, *Pantoea ananatis* e algumas espécies de *Pseudomonas* ainda estavam presentes. Quanto aos parâmetros físico-químicos, a estabilização do pH, da acidez total titulável e da produção de ácidos orgânicos coincidiu com a estabilização da dinâmica das comunidades microbianas e com o predomínio das BAL, a partir do BS4. A maior produção dos ácidos acético, lático e cítrico ocorreu nas primeiras etapas de propagação (BS1-BS4), quando as relações de competitividade e predomínio entre BAL e outros grupos bacterianos estavam sendo estabelecidas. Quanto à metabolização de carboidratos, ambos os *sourdoughs* apresentaram capacidade para reduzir FODMAPs durante as etapas de propagação do fermento, com exceção do SD1 para sorbitol e manitol. Sacarose, frutose e glicose foram completamente consumidos a partir do BS1. Os outros subgrupos de FODMAPs tiveram suas concentrações reduzidas mais tarde, após o BS3 e BS4, coincidindo também com o predomínio das BAL na comunidade microbiana. O subgrupo de polióis teve sua concentração aumentada para o SD1 nas etapas finais da propagação. Durante a fermentação dos pães, a redução de frutanos foi de 69% para o SD1 e 75% para o SD2, no entanto, as concentrações de polióis aumentaram para ambos. Os pães elaborados com *sourdough* apresentaram maiores proporções de ácidos orgânicos e menor concentração de sacarose, frutose, glicose e frutanos que os pães fermentados por *Saccharomyces cerevisiae*. Por outro lado, as concentrações de polióis foram maiores para os pães *sourdough*. No entanto, o aumento na concentração de polióis é muito pequeno em relação ao conteúdo de frutanos, não prejudicando o efeito da fermentação *sourdough* na redução do conteúdo total de FODMAPs.

Considerações Finais

A farinha de trigo foi composta por bactérias pertencentes ao filo *Proteobacteria*, características deste tipo de matéria prima, no entanto, não foram detectadas BAL. A dinâmica microbiana dos *sourdoughs* seguiu as etapas de sucessão microbiana classicamente descritas. A partir do BS1, foram detectadas as primeiras espécies de BAL, provenientes de outras fontes, como o ambiente de produção. A partir do BS4, as BAL (*L. curvatus* e *L. farciminis*) tornaram-se predominantes, sendo capazes de inibir os gêneros *Bacillus*, *Pseudomonas*, *Enterococcus* e o grupo enterobacteria. A partir do BS5, *L. farciminis* tornou-se predominante em ambos os *sourdoughs*, no entanto, a variação de temperatura alterou a composição das populações subdominantes. Ao final das etapas de propagação, no SD1 foram detectadas exclusivamente BAL (*L. farciminis*, *L. curvatus*, *Ln. citreum*

e *Pd. pentosaceus)*. Para o SD2, além de *L. farciminis*, a população sobdominante foi composta por outras bactérias, principalmente pertencentes ao grupo das enterobacterias. Ambas as comunidades microbianas foram capazes de promover a redução do conteúdo total de FODMAPs em pães, principalmente em relação ao subgrupo de frutanos, no entanto, os SD apresentaram capacidades diferentes de metabolização de FODMAPs e ácidos orgânicos durante a fermentação. A temperatura de propagação mostrou-se um fator determinante para dinâmica e composição microbiana do sourdough, o que por sua vez, alterou a atividade metabólica da microbiota durante a fermentação. Os pães elaborados por fermentação sourdough apresentaram concentração significativamente menor de frutanos, sacarose, frutose e glicose que os pães fermentados por *S. cerevisiae* e inferiores às concentrações máximas recomendadas para portadores da SII. Portanto, a fermentação *sourdough* tem potencialidade para ser explorada na obtenção de pães e outros derivados de trigo reduzidos em FODMAPs, representando uma alternativa de consumo para pessoas que possuam SII e/ou SGNC.

Palavras-chave: *Sourdough*. FODMAPs. Frutanos. Bactérias Ácido Láticas. Temperatura. Dinâmica microbiana.

ABSTRACT

The sourdough fermentation has been studied for its ability to improve sensory, nutritional and technological properties of breads. These effects are the result of the metabolism of its microbial community, composed mainly of lactic acid bacteria (LAB) and yeasts. The objective of this study was to characterize the evolution of the microbial community of sourdoughs produced in Brazil during the steps of preparation (propagation) and to investigate the effects of sourdough fermentation on the concentration of FODMAPs (Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols) in breads. FODMAPs are a class of carbohydrates associated with triggering the symptoms of Irritable Bowel Syndrome (IBS) and Non-Celiac Gluten Sensitivity (NCGS). Two temperatures were tested for propagation, 21 °C (SD1) and 30 °C (SD2). The microbial composition of the sourdoughs was investigated by dependent and independent culture methods. The concentration of FODMAPs was evaluated by enzymatic methods and by Liquid Chromatography coupled to Mass Spectrometry (LC/MS-MS). The microbial community of wheat flour was composed exclusively of *Proteobacteria* (*Escherichia hermannii, Kosakonia cowanii, Pantoea ananatis* and some species of *Pseudomonas*). BAL were detected only after the first stage of propagation (BS1) and became predominant (*Lactobacillus farciminis* and *Lactobacillus curvatus*) after the fourth step (BS4). From this step, the difference between the temperatures affected the microbial dynamics between the two treatments. In the end, the microbial community of SD1 was composed exclusively of LAB (*L. farciminis, L. curvatus, Leuconostoc citreum* and *Pediococcus pentosaceus*). *Bacillus, Pseudomonas, Enterococcus* and enterobacteria were gradually inhibited during propagation and were not detected in the final step. On the other hand, the temperature of 30 ºC ± 1ºC favored the presence of atypical bacteria (non-LAB) for SD2. *L. farciminis* and *Escherichia hermannii* were dominant. *Clostridium, Bacillus, Enterococcus* were inhibited, however, *Kosakonia cowanii, Pantoea ananatis* and some species of *Pseudomonas* were still present at the end of the propagation. Regarding FODMAPs, sucrose, fructose and glucose were completely consumed from the first step of fermentation, while the other subgroups of the FODMAPs had their concentrations reduced after the third and fourth steps of fermentation, coinciding with the predominance of LAB in the microbial community. The sourdough breads presented higher proportions of organic acids and a lower concentration of sucrose, fructose, glucose and fructans

compared to breads fermented by *Saccharomyces cerevisiae*. The fructan concentration, the most important subgroup of FODMAPs was 40 to 62% lower in sourdough breads. Therefore, sourdough fermentation represents a promising alternative for the development of breads that can be consumed by IBS- and/or NCGS-sufferers.

Keywords: Sourdough. FODMAPs. Fructans. Lactic Acid Bacteria. Temperature. Microbial Dynamics.

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LIST OF ABREVIATIONS AND ACRONYMS

ACA – Acetic Acid
ACN – Acetonitrile
BAL – Bactérias Ácido-Láticas
BEN – Benzoic acid
BS – Backslopping steps
CFU – Colony-forming unit
CTA – Citric Acid
DNA – Deoxyribonucleic Acid
ESI – Electrospray Ionization
FD – Fermented Dough
FMA - Formic acid
FODMAPs – Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols
FRU – Fructose
GLU – Glucose
HPLC – High-Performance Liquid Chromatography
IBS – Irritable Bowel Syndrome
KES – 1-kestose
LAB – Lactic Acid Bacteria
LCA – Lactic Acid
LCMS/MS – Liquid Chromatography Tandem-Mass Spectrometry
LH-PCR – Length Heterogeneity Polymerase Chain Reaction
MAN – Mannitol
MET – Methanol
mMRS – Maltose Man, Rogosa, Sharpe agar medium
MRM – Multiple-Reaction Monitoring
MYP – Mannitol, Yeast extract, Peptone agar medium
NCGS – Non-celiac Gluten Sensitivity
NYS – 1-nystose
OUT – Operational Taxonomic Units (OTU)
PDA – Photodiode Array Detector (PDA)
pH – Potential of Hydrogen
PHA – Phosphoric acid (PHA
PPA – Propionic Acid
QUIIME – Quantitative Insights into Microbial Ecology
RAF – Raffinose
RNA –Ribonucleic Acid
REP-PCR – Repetitive Element Palindromic-PCR
SBL – Sorbitol

SD1 – Sourdough 1
SD2 – Sourdough 2
SDAM – Sourdough agar medium
SGNC – Sensibilidade ao Glúten Não-Celíaca
SII – Síndrome do Intestino Irritável
SOR – Potassium sorbate
STA – Stachyose
SUC – Sucrose
TTA – Total Titratable Acidity
UFC – Unidade Formadora de Colônia
WFAM – Wheat flour agar medium
YEPD – Yeast Extract-Peptone-Dextrose agar medium

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1. INTRODUCTION

Sourdough is the result of the fermentation of cereal flour and water, by mainly lactic acid bacteria (LAB) and yeasts, which can be developed spontaneously, from the flour and other ingredients, or can be added as a starter culture (VUYST et al., 2014; GOBBETTI et al., 2014; MINERVINI et al., 2014). This fermentation technology is one of the oldest food biotechnologies used to ferment cereals, mainly studied for its effects on the sensory and nutritional properties of bakery products (CHAVAN; CHAVAN, 2011; GOBBETTI et al., 2014).

Many research groups worldwide have focused in reports how the fermentation through sourdough can affect functional features of leavened baked goods, which may increase the bioavailability of minerals (NUOBARIENE et al., 2015) and phytochemicals (KOISTINEN et al., 2016), decrease glycemic response (RINALDI et al., 2017), produce new nutritionally bioactive compounds (ZHAO et al., 2013), among more than ten different health-benefits already reported in the literature (GOBBETTI et al., 2014). In addition to nutritional aspects, the sourdough can extend shelf life (TORRIERI et al., 2014) and improve flavor (REHMAN; PATERSON; PIGGOTT, 2006) and texture (ARENDT; RYAN; DAL BELLO, 2007) of fermented cereal products.

These effects are related on the microbial and enzymatic conversions occurred during the dough preparation (GANZLE, 2014), and dependents on the fermentative microflora, usually comprise heterofermentative LAB, predominantly belonging to the genus *Lactobacillus* and yeasts of the genera *Saccharomyces* and *Candida*. Despite these genres are prevalent, more than 80 species of LAB and 20 yeasts species are typically associated with sourdoughs ecosystems in different countries (GANZLE; RIPARI et al., 2016; VUYST et al., 2016; LHOMME et al., 2015; VRANCKEN et al., 2011; ZHANG et al., 2011).

The metabolism of sourdough microflora comprises conversions, synthesis and degradation of carbohydrates, proteins and lipids, as well as minority components, such as vitamins and phenolic compounds (GANZLE; VERMEULEN; VOGEL, 2007). In relation to carbohydrates, conversions involving hexoses and pentoses and the synthesis of exopolysaccharides are well explored in the literature (GOBBETTI et al., 2005; DERTLI et al., 2016). However, the metabolism of other saccharides remains little known, particularly, its

potential effect on the content of FODMAPS (Fermentable, Oligosaccharides, Disaccharides, Monosaccharides And Polyols), a specific class of short-chain carbohydrates (TUCK et al., 2014) present in most cereals, and presumably susceptible to the action of sourdough LAB and yeasts. FODMAPS intake has been associated with the onset of symptoms of Irritable Bowel Syndrome (IBS). On the other hand, some of these carbohydrates are proven to be prebiotic, contributing to the maintenance of healthy intestinal flora (HALMOS et al., 2014; MUIR et al., 2009). Due to wide range of effects on the gastrointestinal tract, it is important that the role of sourdough fermentation on the FODMAPS content be understood.

This document is organized as follows: Chapter 1 – Literature Review. Some fundamental concepts about sourdough fermentation and its contribution for improving bread properties. Chapter 2 – Effects of sourdough on FODMAPs in bread and potential outcomes on irritable bowel syndrome patients and healthy subjects. Complementary review, published in *Frontiers in Microbiology Journal*, regarding the potential effects of sourdough fermentation on the reduction of FODMAPs and their clinical importance. Chapter 3 – Microbial community Dynamics in Brazilian sourdoughs. Chapter 4 – Use of sourdough fermentation to reducing FODMAPs in breads. Published in the Journal *European Food Research International.*

CHAPTER I - LITERATURE REVIEW

1. BREAD AND SOURDOUGH TECHNOLOGY

Cereal-based foods are important components of the diet, providing mainly carbohydrates, proteins, dietary fiber and micronutrients. Among them is the bread, one of the main foods consumed daily worldwide (HAGER et al., 2012). Bread making is probably one of the oldest technologies known to humankind. Records suggest that Babylonians, Egyptians, Greeks, and Romans used bread as part of their diet long before the A.D. period (CHAVAN; CHAVAN, 2011). According to Vogelmann e Hertel (2011), cereal fermentation has been practiced in European countries for millennia, through the sourdough, a wheat-flour-water mixture, that is fermented to a low pH range due to the metabolic action of yeasts and LAB.

The production of bread using sourdough, involves mixing flour and water in certain proportions and subsequent steps of "backslopping" (or "refreshment"), that consists of inoculating a previously fermented dough (flour–water mixture) with an aliquot of flour and water, repeated before each fermentation step, until the pH is stable. Usually, a sourdough is stable within a week, in daily back-slopped doughs. Then other ingredients are added (flour, water, salt, sugar) and the dough is fermented for hours (CHAVAN; CHAVAN, 2011; HARTH; VAN KERREBROECK; DE VUYST, 2016; VAN DER MEULEN et al., 2007; WECKX et al., 2010).

Originally, bread making required time, characterized by long periods of fermentation. Due to the slow mixing and the long proofing times, bread had traditional characteristics. Nevertheless, bakery industry has undergone a revolution over the past two centuries. Seeking high productivity, the small artisan bakeries gave way for high technological bakery industry, traditional sourdough fermentations were all but replaced by the use of baker's yeast and bread began to be produced using basically *Saccharomyces cerevisiae*. The main consequence of this evolution was a decreased interest for bread flavor through long fermentation processes. However, a new trend is being observed over the last years. Bread flavors became very important as consumers increasingly appreciate and demand the flavor and taste of artisanal breads. The trend in the market is clearly towards tastier breads and an industry adaptation to this demand depends on the incorporation of sourdough fermentation (BRANDT, 2018; DECOCK; CAPPELLE, 2005).

2. THE SOURDOUGH ECOSYSTEM

The applicability of sourdough for bread making requires knowledge about the microbial community composition, since its metabolism will contribute to elucidate the fermentation effects on bread (GÄNZLE; RIPARI, 2016). The standardization of the bread quality also depends on the study of the sourdough microflora.

2.1 SOURDOUGH MICROBIOTA

Many studies have been concerned with isolating and identifying the microbial populations of spontaneous and mature sourdoughs in different parts of the world. More than 80 species of LAB, mostly belonging to the genus *Lactobacillus*, were found in different sourdoughs worldwide. *Leuconostoc*, *Lactococcus*, *Enterococcus*, and *Weissella* are also detected frequently. Regarding yeasts, more than 20 species have already been isolated, mainly *Saccharomyces*, *Candida*, and *Kazachstania* (DE VUYST et al., 2016; GOBBETTI et al., 2016; SIEPMANN et al., 2018; VAN KERREBROECK; MAES; DE VUYST, 2017).

A sourdough community usually comprises associative bacteria and yeasts, and a stable number of yeasts and LAB has been observed in mature sourdoughs (VRANCKEN et al., 2011), sustaining in relations of competitiveness and associations. It is generally considered that the ratio of LAB to yeast should be 100:1 for optimal activity (GOBBETTI et al., 2016). A single sourdough may harbor simple (two species) to rather complex (more than six species) microbial population at a given time (MINERVINI et al., 2014), among which one or two species will be yeasts (DE VUYST et al., 2016; VRANCKEN et al., 2010).

The obligately heterofermentative *Lactobacillus sanfranciscensis*, *Lactobacillus brevis*, *Lactobacillus reuteri*, *Lactobacillus fermentum*, and *Leuconostoc citreum*; the facultatively heterofermentative *Lactobacillus (par)alimentarius*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus*, and finally, the obligately homofermentative *Lactobacillus amylovorus* and *Lactobacillus acidophilus* (DE VUYST et al., 2014) have been found more frequently. In a recent review (DE VUYST et al., 2016), the yeast species diversity of different bakery sourdoughs was analyzed. The six most encountered species were *S. cerevisiae*, *Candida humilis*, *Torulaspora delbrueckii*,

Wickerhamomyces anomalus, *Kazachstania exigua*, and *Pichia kudriavzevii*. For most sourdough ecosystems, heterofermentative LAB species are predominant. On the other hand, many sourdoughs contain associations of hetero and homofermentative species. Heterofermentative species may be dominant because of their high adaptability, but there are several parameters determinants for the natural selection of the microflora, that are discussed in the next section.

2.2 MICROBIAL DYNAMICS OF SOURDOUGH COMMUNITY

The microbial population is not always the same for a given sourdough, but evolves from the first fermentation and through the consecutive backslopping steps until the stabilization. This evolution is reported as the microbial dynamics, and results in both succession of microbial populations (and strains) and alteration of metabolic patterns. The first microbial consortia of sourdough corresponds to the autochthonous microorganisms of flour, consisting of LAB, aerobic bacteria, *Enterobacteriaceae*, yeasts and moulds (SCHEIRLINCK et al., 2007), not exceeding 5 log CFU/g (VAN DER MEULEN et al., 2007).

Over the consecutive fermentation steps, taking into account physic-chemical characteristics like water activity (aw), pH and redox potential, and the ability to use of the available substrates, sourdough allows LAB and yeast to outgrow other microbial populations (MINERVINI et al., 2014). These aspects will determine which species will predominate and which metabolites will be produced.

Several factors will drive the dynamics of the sourdough microbial community. Many parameters are involved in the selection and maintenance of the microbial structure of a given sourdough over time. These factors influence the function of sourdough microbiota as well as the most of the metabolic traits that determine competitiveness and consequently, the composition of a given sourdough, also directly contribute to product quality (GÄNZLE; RIPARI, 2016; MINERVINI et al., 2014), which makes sourdough a complex and dynamic ecosystem.

The drivers for the establishment and composition of the sourdough microbiota are categorized into exogenous and endogenous, distinguishing between technological parameters and parameters that are not fully controllable, considering, above all, that the microbial growth is influenced by multiple combinations of them (VAN DER MEULEN et al., 2007). Comprehensive interpretation of such factors is relevant to

standardize the sourdough performance and to allow its propagation and use more control and safe (GOBBETTI et al., 2016).

Sourdough fermentation requires continuous propagation or back-slopping, which has relatively controlled conditions of time, temperature and ingredients. The diversity of processes - for example, number of back-slopping cycles - and raw materials employed in sourdough fermentation explain the diversity of sourdough microbiota, in combination with other factors, like differences in the composition and enzyme activities between grains harvested in different years, can help describe the dynamics of microbial communities (GÄNZLE; RIPARI, 2016). According to Minervini et al. (2014), technological parameters includes dough yield; % of sourdough used as inoculum; content of NaCl; the redox potential; time and temperature of fermentation; number of backslopping, while the not controllable aspects comprises flour and house microbiota of ambient.

The study of the metabolites produced during the backslopping stages is relevant to explain why certain sourdough species became dominant during spontaneous sourdough fermentation (VAN DER MEULEN et al., 2007). The composition of microbiota is predominantly shaped by the selection of competitive organisms, like *Lb. sanfranciscensis*, a key species of sourdoughs microbiota worldwide (GÄNZLE; RIPARI, 2016). For example, the capacity to ferment all four carbohydrates contained in wheat flour represents an important advantage (DE VUYST; NEYSENS, 2005).

A stable number of yeasts and LAB has been observed through sourdough fermentations (VRANCKEN et al., 2011), sustaining in relations of competitiveness and associations. In sourdoughs, microorganisms have to withstand a specific and stressful environment, characterized by a low pH, low oxygen tension, and carbohydrates (mainly maltose) that have to be shared (DE VUYST et al., 2014). The best-known association of yeasts and LAB in sourdough is that of the maltose-negative yeasts *K. exigua* or *C. humilis* with the maltose-positive *Lb. sanfranciscensis*, thus avoiding nutritional competition. This interaction is further enhanced by excretion of glucose upon maltose consumption through by *Lb. sanfranciscensis*, which can then serve as an energy source for the maltose-negative yeasts (VRANCKEN et al., 2010).

Although more research is needed to verify whether the species diversity encountered in baking environments worldwide is sourdough-specific or not, the extensive research carried out by Gobbetti et al.

(2016) showed that the dominating yeast species were those with greater competitive capacity, or strains that had high adaptability to the environment, as reported by Vrancken et al. (2010), when *W. anomalus* developed, during the experiment, the ability to withstand low pH. The species that are predominant were capable of assimilating various carbohydrates, and growth at acid pH.

The microflora modulation usually occurs during a few days in sourdoughs daily backslopped. A sourdough with constant cell densities of LAB and yeasts, acidification and leavening capacities, may be achieved within a week, reaching an equilibrium through a three-step process: (i - from the start until day 2) prevalence of species that were not specific for sourdough, such as those belonging to the genera *Enterococcus*, *Lactococcus*, and *Leuconostoc*, (ii - days 2 to 5) prevalence of sourdough-typical species, such as *Lactobacillus*, *Pediococcus*, and *Weissella* and (iii – 5 to 7 days) prevalence of well-adapted sourdough-typical strains, i.e., *Lb. fermentum* and *Lb. plantarum*. Both the composition and the metabolic activity of the microbiota remained unchanged once a stable ecosystem is achieved (VAN DER MEULEN et al., 2007).

The dynamics of yeasts during backslopping is little known. While most workers seek to characterize the bacterial community, few studies on sourdough yeast species diversity and identification are available. Probably, due to the fact that wild-type yeasts have almost been replaced by the use of *S.cerevisiae* as a starter culture. The widespread occurrence of *S. cerevisiae* in spontaneous sourdoughs may be linked to the presence of baker's yeast in the bakery environment. Moreover, the ability of all *S. cerevisiae* and *W. anomalus* to strongly assimilate maltose and sucrose, the major carbohydrates of flour, might be considered as an advantage over others species of yeasts (VRANCKEN et al., 2010).

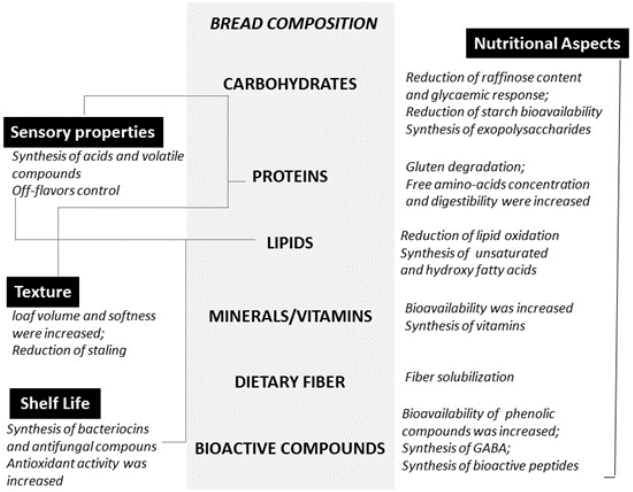
The population dynamics of microbial food ecosystems have been studied mainly through culture-dependent and –independent methods. Both methods have some limitations. For example, in traditional plating methods, the culture media could be not appropriate for recover all species present. On the other hand, some strain may have lower loads than the detection limit by independent methods. Results obtained by both methods displayed a more complete profile of the sourdough ecosystem, confirming and complementing each other (LIU et al., 2016).

In the last years, meta-analysis methods, such as metagenomics and metatranscriptomics, have become ubiquitous in the field of ecosystem exploration. The use of pyrosequencing and the parallelized ligation-mediated and bead-based sequencing, and derives methods such as high-throughput sequencing of genes (HTS) were considered as the "Next-Generation Sequencing" techniques (NGS), will allow the expanse of knowledge about diversity of complex microbiomes, like sourdoughs. The availability of new and affordable molecular techniques to characterize microbial flora has aroused interest regarding the potential to overcome classical microbiology limitations, or more accurately, to complement traditional microbiology with culture-independent strategies (KERGOURLAY et al., 2015). The combination of these different approaches will allow the study of the functionalities and dynamics of the microbial community

3. IMPROVING BREAD PROPERTIES

Sourdough fermentation is known to contribute in many ways to the enhanced nutritional and technological qualities of bakery products. Moreover, microbial activities of both yeasts and LAB can result in the production of health-improving metabolites, increasing globally quality of breads (figure 1).

Figure 1. Potential effects of sourdough fermentation on bread characteristics.



The differentiation between sourdough and straight-doughs (breads produced with *S. cerevisiae* and fermented for short periods of time) is based on the contribution of microbial community to metabolic conversions at the dough stage (GÄNZLE; RIPARI, 2016). LAB and yeasts have been intensively studied with respect to their metabolism, which includes macro and micronutrients. After the first backslopping, complex carbohydrates, mainly starch, are quickly hydrolyzed to mono/di-saccharides (maltose, fructose and glucose), by flour and microbial amylases. Proteins are hydrolyzed to more easily usable nutrients (peptides and free amino acids) by proteases (MINERVINI et al., 2014). Simultaneously and subsequently, these and other metabolic pathways of the fermentative flora occur, modifying the dough.

3.1 CARBOHYDRATES

Carbohydrates are the major components of cereals. At maturity, the wheat grain consists of 85% (w/w) carbohydrate, 80% of which is starch; approximately 7% low molecular mass mono-, di-, and oligosaccharides and fructans; and about 12% cell wall polysaccharides (SHEWRY; HEY, 2015). Certainly, its metabolism is of great importance for the characteristics of cereals-based fermented foods. Not by chance, the more expressive metabolic activities of the microbial communities are acidification (LAB), leavening (yeasts and heterofermentative LAB species), and flavor formation (LAB and yeasts) (HARTH; VAN KERREBROECK; DE VUYST, 2016), all related to the conversion of carbohydrates.

Besides conversion of carbohydrates to organic acids, which is inherent in the fermentation process, the action of sourdough LAB and yeast comprises the reduction of starch digestibility (RINALDI et al., 2017), the exopolysaccharide production from sucrose and the use of alternative electron acceptors (GÄNZLE; RIPARI, 2016).

Carbohydrate utilization and organic acid production. Flavor, texture and shelf life are improved from the most basic metabolism associated with fermentation (GÄNZLE; RIPARI, 2016). The primary metabolic products of homofermentative LAB are lactic and acetic acids with lesser amounts of citric and malic acids. Thereafter, many other flavor and flavor compounds may be produced by yeast and heterofermentative LABs (SALIM-UR-REHMAN; PATERSON; PIGGOTT, 2006). Organic acids affect the protein and starch fractions

of flour, additionally, the drop in pH associated with acid production enhances the activity of proteases and amylases, thus delaying staling (BARTKIENE et al., 2017). Whereas acetic acid and lactic acid both contribute to the acidity and texture forming components of sourdough (AREND; RYAN; BELLO, 2007), acetic acid has important role in flavor (PÉTEL; ONNO; PROST, 2017) and shelf life, due to antifungal activity (DAGNAS et al., 2015).

Starch bioavailability. The synthesis of organic acids, especially lactic acid has been related to the decrease of the digestibility rate of the starch (GOBBETTI et al., 2014). In association with the synthesis of acetic and propionic acids, able of lowering the gastric emptying rate, such the more interaction between starch and cereal proteins, that reduce the starch bioavailability, can help explain why the sourdough fermentation has the ability to reduce glycaemic index of breads (GOBBETTI et al., 2014; NOVOTNI et al., 2012).

Synthesis of Exopolysaccharides (EPS). EPS are exogenous microbial biopolymers produced by several LAB species that may be assembled as capsular forms in with the cell surface or be secreted in their environment (DERTLI et al., 2016; KETABI et al., 2008). Approximately 30 species of lactobacilli are described as EPS producers, among them, the best known are *Lactobacillus casei*, *Lb. brevis*, *Lactobacillus curvatus*, *Lb. rhamnosus* and *Lb. plantarum* (BADEL; BERNARDI; MICHAUD, 2011), strains associated with the sourdough ecosystem. The production of EPS in the sourdough by LAB breads improve texture properties and delays the staling of bread, extending shelf life of breads as well as improve consumer's health, since that compounds are considered prebiotics (DERTLI et al., 2016; KETABI et al., 2008; TORRIERI et al., 2014). Moreover, the microbial EPS synthesized during sourdough fermentation is considered as promising tool to improve texture of gluten-free products, produced with cereals which do not contain gluten (CAMPO et al., 2016, GOBBETTI et al., 2014).

Use of alternative electron acceptors. Fructose is used as an alternative external electron acceptor by the strictly heterofermentative LAB species; its reduction to mannitol shifts ethanol formation to acetate production (GOBBETTI et al., 2005). The use of citrate as electron acceptor too increased acetate and lactate levels (DE VUYST et al., 2009). Acetate (and other esters like lactate) contributes to the typical aroma of sourdoughs (PÉTEL; ONNO; PROST, 2017).

Minimization of acrylamide content. Metabolism of carbohydrates during fermentation affects the formation of acrylamide. *Lb. plantarum* sourdough significantly decreased the acrylamide content in bread samples, which was correlated to acidification and the activity of LAB amylolytic enzymes (BARTKIENE et al., 2017).

All these metabolic pathways are already well documented in the literature. However, sourdough has potentials not yet explored in relation to some carbohydrates, especially regarding a specific group named FODMAPs. FODMAP is an acronym for Fermentable Oligo-, Di- and Mono-saccharides And Polyols, a class of compounds, predominantly short-chain carbohydrates, poorly absorbed in the small intestine and rapidly fermented by bacteria in the large intestine (ZANNINI, ARENDT, 2018).

FODMAPs and the Irritable Bowel Syndrome (IBS) There is stronger evidence supporting the efficacy of a low FODMAPs diet for the management of IBS than for any other diet therapy (MUIR et al., 2019). The sourdough fermentation has the potential to lower the quantity of FODMAPs, mostly the fructans fraction, to levels that can be well tolerated by individuals with IBS. In this context, sourdough breads can constitute an excellent alternative for IBS-sufferers, due to the ability of LAB and yeast to degrading FODMAPs during the fermentation, as is better discussed in the review published and presented in chapter II of this thesis.

3.2 PROTEIN

Proteolysis occurs during sourdough fermentation is one of the key aspects that affect the overall quality of sourdough bread. Flavor and texture are strongly modified, the amount of free amino acids are increased, the content of allergenic compounds can be reduced and gluten proteins can be partially degraded (GOBBETTI et al., 2014). The proteolytic events are observed in two moments. First, the microbial acidification shifts the dough pH to optimum of the cereal proteinases (primary proteolysis). The acidification and the reduction of disulfide bonds in gluten proteins by heterofermentative lactobacilli degrade the gluten to smaller peptides, disrupting the gluten network and increasing the solubility of the gluten proteins. The hydrolysis of peptides (secondary proteolysis) by sourdough lactobacilli accumulates amino acids in dough whereas the activity of yeasts decreases amino acids

levels during growth and increase when the yeast growth has ceased (GÄNZLE; LOPONEN; GOBBETTI, 2008).

Accumulation of free amino acids. The capacity of yeasts and LAB to deliver free amino acids and hydrolyze them in secondary proteolysis to aldehydes or the corresponding alcohols increases protein bioavailability (NIONELLI; RIZZELLO, 2016) and contributes to the formation of taste and aroma compounds (PÉTEL; ONNO; PROST, 2017). The presence of free amino acids favors the Maillard Reaction during baking of bread, and therefore the production of compounds has a striking effect on the flavor of bread (SALIM-UR-REHMAN; PATERSON; PIGGOTT, 2006). The synthesis/release of peptides and amino acids can help to regulate the glucose metabolism, in association of other mechanisms (GOBBETTI et al., 2014).

Celiac disease. Celiac disease (CD) is a chronic immune-mediated small bowel enteropathy resulting from gluten exposure. Prevalence is estimated at approximately 1% of the population, genetically susceptible individuals (BUTTERWORTH; LOS, 2019). The sourdough has recently been tested in order to obtain gluten-free wheat breads. The acidification of bread dough from the production of organic acids associated with the action of the LAB proteases has shown potential to degrade gluten, even in relation to the peptide 33-mer, considered the most immunogenic peptide involved in celiac disease (ALVAREZ-SIEIRO et al., 2015; GEREZ et al., 2012; RIZZELLO et al., 2007; RIZZELLO et al., 2014). For now, this is a hypothesis that needs further studies, given the congruity of the disease and the fact that gluten degradation is a strain-specific activity and depends on the selection of strains capable of degrading gluten (MUIR et al., 2019).

The Non Celiac Gluten/Wheat Sensitivity (NCGS/WS). The NCGS/WS is an emerging clinical condition, which is part of the spectrum of gluten-related disorders also closely related to IBS, characterized by a wide array of both gastrointestinal and extra-intestinal symptoms. By definition, the NCGS/WS is a reaction to gluten in the absence of allergic and autoimmune mechanisms. However, this condition may not triggered by gluten at all, since other components of wheat and other gluten-containing grains could be responsible for the onset of symptoms (HENGGELER;VERÍSSIMO; RAMOS, 2017). The implication of FODMAPs in the exacerbation of the symptoms of this condition are still very recent and not conclusive, however, there are evidences that the NCGS can also related to FODMAPs intake (BIESIEKIERSKI et al., 2013; MUIR et al. 2019). Due to the extensive

degradation of gluten proteins and FODMAPs, sourdough has been suggested as a new tool for production of breads to meet this nutritional demand, since fermentation can reduce gluten and FODMAPs levels to an extent that these products are tolerated by sensitive patients.

Synthesis of bioactive peptides. Derived from proteins by the action of microbial and plant proteolytic enzymes, numerous bioactive peptides have been found in sourdough, among them, several antioxidants (GOBBETTI et al., 2014), antifungal (RIZZELLO et al., 2011) and antihypertensives (OMEDI et al., 2016). Active concentrations of γ- aminobutyrate (GABA), a bioactive metabolite of glutamate, were found in breads submitted to sourdough fermentation (BHANWAR et al., 2013; CODA; RIZZELLO; GOBBETTI, 2010).

3.3 LIPIDS

Lipids are only a minor component of wheat and rye flours but have a significant effect on bread quality (GÄNZLE; VERMEULEN; VOGEL, 2007). Two different processes involving lipids in sourdough have already been reported: control of lipid oxidation and production of antifungal lipids.

Control of lipid oxidation. Enzymatic and microbial conversion of fatty acids by endogenous lipoxygenase of cereals can result in metabolites with undesirable taste and aroma (GÄNZLE, 2014). Hydrogen peroxide formation by homofermentative lactobacilli and some yeast can enhance lipid oxidation and the formation of aldehydes during sourdough fermentation (VERMEULEN et al., 2007). However, obligate heterofermentative lactobacilli and *S. cerevisiae* can decrease the oxidation of sourdoughs through aldehyde dehydrogenases (GÄNZLE; VERMEULEN; VOGEL, 2007).

Synthesis of antifungal hydroxyl fatty acids. The production of antifungal hydroxy fatty acids from linoleic acid by *Lb. sanfranciscensis*, *Lb. plantarum*, and *Lb. hammesii* was described by Black et al. (2013). The application of antifungal metabolites generated was able to increase the mold-free shelf life from two to more than six days. Combined with the other antifungal compounds presents in sourdough, already reported, the effects of antifungal compounds produced by LAB and yeasts have the potential to reduce the levels of chemical additives needed in the bakery industry. Sourdough fermentation with *Lb. plantarum* strains was investigated by Ryan; Bello e Arendt (2008) for the ability to inhibit growth of common bread

spoilage fungi. In both in vitro and sourdough wheat bread system, the antifungal sourdoughs significantly affected the outgrowth of *Aspergillus niger*, *Fusarium culmorum*, and *Penicillium expansum* spores.

3.4 VITAMINS, MINERALS AND PHYTOCHEMICALS

Yeast metabolism encompasses the capacity to produce several vitamins, in particular those of the vitamin B complex (DE VUYST et al., 2016). LAB may also affect the contents of vitamins, but the increase during sourdough fermentation is due mainly to yeasts. *Candida milleri, S. cerevisiae* and *T. delbrueckii* were able to produce folate during fermentation of rye sourdoughs more than lactobacilli strains (KARILUOTO et al., 2006). *S. cerevisiae* and sourdough fermentation increased the content of riboflavin in wheat breads, while pyridoxine levels decreased. The highest levels of B vitamins were achieved by long yeast fermentations (BATIFOULIER et al., 2005).

During baking of rye sourdough bread, the concentration of thiamine, nicotinic acid, pyridoxal and riboflavin decreased, while the content of nicotinamide increased ten-fold, presumably due to microbial activity during sourdough fermentation (MIHHALEVSKI et al., 2013). The numerous microbial activities and technological parameters may affect the content of vitamins in breads, increasing or decreasing it. In this way, the action of LAB and yeast fermentation on vitamins should be more clearly investigated (GOBBETTI et al., 2014). Nevertheless, the potential of certain strains, especially yeasts, to increase the concentration of vitamins can be explored by the process control and the selection of specific strains.

Increase mineral bioavailability by reduction of phytate can be achieved by enzymatic degradation during bread making, by activity of endogenous phytase or by action of phytase-active LAB and yeasts. More than 90% of IP6 was hydrolysed during 24 h dough fermentation with *Lb. fermentum* (NUOBARIENE et al., 2015). The higher activity of phytase of sourdough microorganisms raise the concentration of free minerals, like iron, zinc, calcium, potassium and magnesium (RIZZELLO et al., 2010). These enzymes are able to dephosphorylate phytate, forming free inorganic phosphate and inositol phosphate esters, which have less capacity to influence mineral solubility and bioavailability. Phytase activity is accelerated in the acidic environment

produced in sourdough fermentation (POUTANEN; FLANDER; KATINA, 2009).

Changes of different phytochemicals such as phenolic compounds were observed in sourdough fermentations (GOBBETTI et al., 2014). Sourdough fermentation by *Lb. rhamnosus*, *Lb. plantarum*, *Lb. rossiae* and *S. cerevisiae* increased the total phenolic content and enhanced antioxidant activity (DORDEVIĆ; ŠILER-MARINKOVIĆ; DIMITRIJEVIĆ-BRANKOVIĆ, 2010; RIZZELLO et al., 2016). Moreover, LAB and yeasts can add to the antioxidant capacity of cereal products through their effects on the availability of phenolic compounds (WANG; HE; CHEN, 2014).

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CHAPTER II - EFFECTS OF SOURDOUGH ON FODMAPS IN BREAD AND POTENTIAL OUTCOMES ON IRRITABLE BOWEL SYNDROME PATIENTS AND HEALTHY SUBJECTS

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EFFECTS OF SOURDOUGH ON FODMAPS IN BREAD AND POTENTIAL OUTCOMES ON IRRITABLE BOWEL SYNDROME PATIENTS AND HEALTHY SUBJECTS

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Background. Fermentable, Oligosaccharides, Disaccharides, Monosaccharides And Polyols (FODMAPs) are an heterogeneous group of compounds that can be poorly digested and may have a range of effects on gastrointestinal processes. FODMAPs are found in a wide variety of foods, including bread. FODMAPs' intake is associated with the onset of symptoms of Irritable Bowel Syndrome (IBS). On the other hand, some FODMAPs contribute to the healthy maintenance of intestinal microbiota. Volume increase of bread dough commonly relies on the use of two biological leavening agents, sourdough and baker's yeast and, in some cases, a combination of both.
Scope and Approach. The main objective of this review is to discuss the association between FODMAPs and IBS, beneficial effects of FODMAPs on healthy subjects and potential impact of biological leavening agents on FODMAPs content of bread.
Key Findings and Conclusion. Given that yeasts and lactic acid bacteria, the dominant microorganisms in sourdough, may degrade FODMAPs, it would be possible to modulate the FODMAPs concentration in bread, thus positively affecting consumers' health.

Keywords: Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols; Irritable Bowel Syndrome; bread; lactic acid bacteria; yeasts; sourdough.

1. INTRODUCTION

Nowadays, tailored nutritional recommendations may be designed in order to treat or prevent diseases (Betts & Gonzalez, 2016). For instance, lactose- and gluten-containing foods must be avoided by subjects suffering from hypolactasia and celiac disease (De Toro-Martín et al., 2017). Fermentable, Oligosaccharides, Disaccharides, Monosaccharides And Polyols (FODMAPs) are an heterogeneous group of compounds (most of which are short-chain carbohydrates) that can be poorly digested and may have a range of effects on gastrointestinal processes. This group includes lactose, fructose in excess of glucose, fructans and fructooligosaccharides (FOS, such as nystose and kestose), galacto-oligosaccharides (GOS such as raffinose and stachyose), and sugar polyols (sorbitol, mannitol) (Muir et al., 2009).

FODMAPs are found in a wide variety of foods. Their dietary uptake mostly results from honey and fruits as watermelons, pears, and apples (fructose); milk and dairy products (lactose); rye, wheat, artichoke, garlic, onions, and broccoli (fructans and FOS); pulses (GOS); stone fruits and artificial sweeteners, mushrooms, broccoli, and cauliflower (sugar polyols) (Muir et al., 2009; Shepherd, et al., 2013).

Fructans and FOS are the main FODMAPs in wheat-based products (Biesiekierski et al., 2011; Shewry & Hey, 2015; Ziegler et al., 2016). They are composed of fructose molecules linked to each other through fructosyl-fructose bonds and of one sucrose terminal end. Inulin-type and levan-type are linear fructans containing β-(1/2) and β-(6/2), respectively, fructosyl-fructose bonds. Both bonds are found in branched chain fructans called “graminans” (Verspreet et al., 2015). These bonds may be cleaved by several enzymes: endo-inulinase, exo-β-fructosidase, endo-levanase, β-fructofuranosidase (alias invertase), and levansucrase. Endo-inulinase and endo-levanase act on internal linkages, generating oligosaccharides with a lower degree of polymerization. Exo-β-fructosidase and β-fructofuranosidase liberate terminal fructose. Invertase, typically splitting sucrose into glucose and fructose, could act on fructans and FOS (Verspreet et al., 2013). Levansucrase generates sucrose from FOS and could further split sucrose into glucose and fructose (De Angelis et al., 2017). GOS are soluble, non-reducing α(1,6)

galactosyl extensions of sucrose. From a chemical point of view, it would be more correct to name these FODMAPs as "Raffinose Family of Oligosaccharides", but they are usually referred to as "GOS". Elongation of the trisaccharide raffinose with galactose residues leads to stachyose and verbascose (Van Den Ende, 2013). α-galactosidase acts on the α-1,6 linkage, thus hydrolyzing GOS (Katrolia et al., 2014). In addition, inulinase, invertase and levansucrase may cleave the bond between fructose and glucose in sucrose, raffinose, stachyose, and verbascose to yield glucose, melibiose, manninotriose, and manninotetraose, respectively. Thus, the combined action of α-galactosidase and enzymes cleaving the fructose-glucose bond converts non-digestible GOS to carbohydrates with lower polymerization degree and potential prebiotic activity (Morel et al., 2015; Teixeira et al., 2012).

FODMAPs' intake may be detrimental for health, being associated, for instance, with the onset of symptoms of Irritable Bowel Syndrome (IBS) (Barret, 2017). On the other hand, some FODMAPs are proven to be prebiotic, contributing to the healthy maintenance of intestinal microbiota (Halmos et al., 2014; Muir et al., 2009). Wheat-based products (e.g., bread, breakfast cereals, pasta) account for a major part of daily consumed FODMAPs, due to their high consumption as staple foods worldwide (Verspreet et al., 2015). In particular, since ages of ancient civilizations (Babylonians, Egyptians, Greeks, and Romans), bread has been a major component of daily diet of several peoples (Chavan & Chavan, 2011). Volume increase of dough (flour and water) usually occurring during bread-making commonly relies on the use of biological leavening agents. Sourdough is the traditional leavening agent, resulting from the fermentation of cereal flour and water, by mainly lactic acid bacteria (LAB) and yeasts (De Vuyst et al., 2014; Gobbetti et al., 2014; Minervini et al., 2014).

Starting from the beginning of the twentieth century, sourdough was gradually replaced by baker's yeast (consisting of cell biomass mainly belonging to the yeast species *Saccharomyces cerevisiae*), a microbial starter culture produced at industrial level and distributed to bakers, which add it at low percentages (0.5 – 2.5%) in bread dough to obtain leavening. The main consequence of this revolution was a decreased flavor, due to reduced fermentation times and almost exclusive yeast metabolic activities (Cauvain & Young, 2009). It has also been hypothesized that short fermentation may have contributed to bread intolerance through its effects on fermentation in the colon (Costabile et al., 2014). However, during the two last decades, an

increasing number of consumers is demanding traditional breads endowed with higher aroma and taste and the main industrial reply consists in the rediscovery of sourdough fermentation (Cauvain &Young, 2009; Decock & Cappelle, 2005). This review focuses on the association between FODMAPs and IBS, beneficial effects of FODMAPs on healthy subjects and potential impact of biological leavening agents on FODMAPs content of bread.

2. IBS, FODMAPS AND DIET

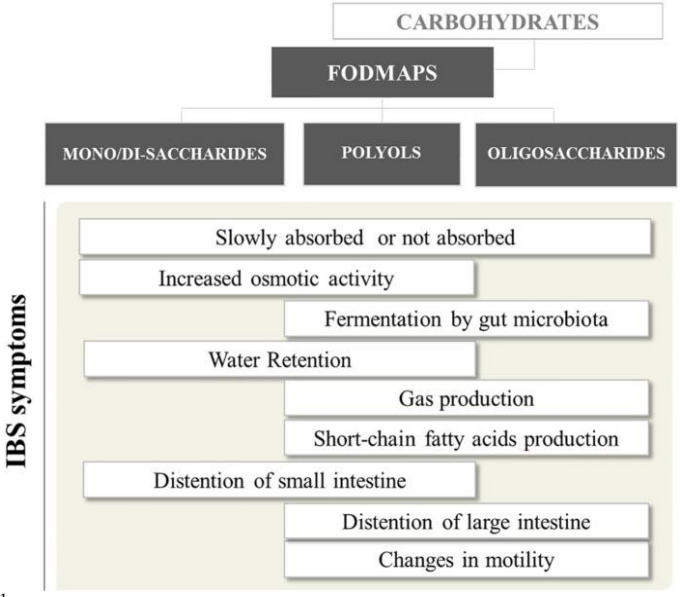
IBS is one of the most common type of functional bowel disorders, in which abdominal pain is associated with a change in bowel habits (Giorgio et al., 2015). Symptoms include diarrhea, constipation, bloating, distension, abdominal discomfort and/or pain and flatulence (Marsh et al., 2016; Tuck et al., 2014). The prevalence of IBS is estimated between 7 and 21% worldwide (Brandt et al., 2009; Lovell & Ford, 2012).

For some individuals the intake of FODMAPs may lead to an exacerbation of symptoms associated with IBS and other functional gut disorders (Gibson et al., 2015). The triggering of IBS symptoms by the consumption of FODMAPs is attributed to their slow or partial absorption in the small intestine. These carbohydrates are described as ‘fermentable’ because they are likely to be fermented in the colon due to the absence, or reduced concentration, of hydrolase or because they are not absorbed completely (Staudacher et al., 2012). The gastro-intestinal tract has no hydrolase capable of degrading fructans or GOS (Fedewa & Rao, 2014; Kolida & Gibson, 2007; Staudacher et al., 2014; Teixeira et al., 2012). Polyols are only partially digested and absorbed in the small intestine and reach the colon, where they are fermented by bacteria (Murillo, Arévalo, & Jáuregui, 2016). In the small intestine, free fructose is absorbed thanks to GLUT5 and GLUT2 transporters. GLUT5 is a facultative transporter specific for fructose, and provides carrier-mediated facilitated diffusion. Although present along the whole length of the small intestine, GLUT5 shows low capacity leading to slow uptake of luminal fructose. GLUT2 is a low-affinity, facultative transporter for glucose, fructose and galactose, that is activated in presence of high luminal glucose concentrations. Although fructose absorption is markedly enhanced in presence of luminal free glucose, if the latter is present in excess, the risk of fructose malabsorption is greater, because of the low affinity characterizing GLUT2. Other factors

have been shown to alter fructose absorption, but the underlying mechanisms have not been totally elucidated (Gibson, 2007).

Delivery of FODMAPs to the lumen of the distal small intestine and proximal large intestine may have adverse consequences in IBS patients (Fig. 1). FODMAPs are believed to induce symptoms by two main mechanisms: (i) drawing water into the small intestine, causing distension, swelling, and discomfort; (ii) the rapid fermentation of FODMAPs would generates gas, distending the colon and causing flatulence, swelling and discomfort.

Figure 1. Short-chain carbohydrates and their relevance to gut health[1].



[1]Bars more shifted to the left or right = effects attributed mostly to mono-disaccharides/polyols or oligosaccharides, respectively.

The first mechanism is mostly related to low molecular mass FODMAPs (e.g., fructose), which are osmotically active in the intestinal lumen. Therefore, volume of fluid entering the bowel increases, and this may provide a natural laxative effect in healthy people, but may contribute to diarrhea in IBS-sufferers. Regarding the second mechanism, intestinal bacteria rapidly ferment FODMAPs (e.g., FOS) releasing short-chain fatty acids (SCFA) and gases (e.g., carbon dioxide,

hydrogen, methane) (Giorgio et al., 2015). Hence, FODMAPs malabsorption and their fermentation in the proximal colon can add gas and water to the luminal contents, leading to distension and the onset of other symptoms (Ong et al., 2010). Moreover, patients with visceral hypersensitivity respond exaggeratedly to gas and fluid distension caused by malabsorption of carbohydrates (Major et al., 2017). Although all FODMAP have similar physiological effects, each FODMAPs subgroup has a different gastrointestinal response, depending on molecular mass, rapidity of absorption and osmolarity (Murray et al., 2014). Overall, slowly absorbed fructose and polyols have a greater osmotic effect per molecule than fructans. Conversely, oligosaccharides, being scarcely absorbed across the small intestine, will have greater fermentative effects than fructose and polyols (Giorgio et al., 2015).

A limited number of effective treatment strategies is available for IBS (Tuck et al., 2014). Modulation of FODMAPs' intake in volume and type could be a means for controlling gut symptoms (Gibson et al., 2015); that is why it is extremely necessary to know the concentration of FODMAPs present in the greatest possible number of foods. Control of FODMAPs ingested as food components is known as "low FODMAPs diet" (Marsh et al., 2016). The recommended total daily intake of FODMAPs in patients with IBS ranges from 5-30 g per day (Böhn et al., 2015; Staudacher et al., 2012). The reduction of gastrointestinal and systemic symptoms by restriction of FODMAPs ingestion has been observed by many research groups (Barrett, 2017; Böhn et al., 2015; Pedersen et al., 2014; Roest et al., 2013). The dietary manipulation of FODMAPs ingestion was able to impact on the total amount of gastrointestinal gas production and the spectrum of gas produced (hydrogen *vs* methane) in healthy individuals and in patients with IBS (Ong et al., 2010). Patients subjected to diets containing low amount of FODMAPs present milder symptoms of IBS, through dietary restriction and re-challenges tests, used to determine individual tolerance to various short-chain carbohydrates (Halmos et al., 2014; Tuck et al., 2014).

The adoption of a low-FODMAPs diet presents some difficulties related to the lack of clear "cut-off levels" for FODMAPs content in foods and no available information on FODMAPs content on food packages. The definition of cut-off values should consider the amount of each particular FODMAPs present in a food, the typical serving size of food consumed in a single sitting, and the commonly triggered symptoms in individuals with IBS. To build a comprehensive

composition database for FODMAPs in food, based on "ad hoc" studies, is essential for defining cut-off values (Muir et al., 2009). In addition, in long term, the low FODMAPs diet could change the gastrointestinal microbiota (Halmos et al., 2014; Tuck et al., 2014). Changes in total bacteria abundance, relative abundance of bifidobacteria (Staudacher et al., 2012) and strongly butyrate producing clostridial groups or *Akkermansia muciniphila*, positively associated with health, were described (Halmos et al, 2014). Moreover, the low FODMAPs diet could reduce the intestinal production of SCFA (Ong et al., 2010).

3. BENEFICIAL EFFECTS OF FODMAP

FODMAPs are fermented in the colon to SCFA, which exert multiple beneficial effects on human health. Apart from being a major energy source for colonocytes, SCFA play a major immunological role in the gut, and help various physiological functions including colonic mobility and blood flow, and gastrointestinal pH, which can influence uptake and absorption of electrolytes and nutrients (Den Besten et al., 2013; Tan et al., 2014). Furthermore, inulin-type fructans and GOS have been proposed for the treatment of metabolic endotoxaemia or low-grade inflammation in overweight/obese people (Fernandes et al., 2017; Morel et al., 2015). Some FODMAPs are being increasingly used in food industry as prebiotics, either as bioactive ingredients or as supplements, to promote colonic health. This kind of food/supplements should clearly be avoided by IBS patients. The higher FODMAPs' intake compared with that of the low FODMAPs or habitual diets was associated with specific stimulation of bacterial groups with putative health benefits (Halmos et al., 2014).

4. HOW BREAD-MAKING MAY CHANGE FODMAPS LEVEL

The FODMAPs content of breads depends on the nature of the grain-ingredient, as well as on the processing parameters (Biesiekierski et al., 2011). Despite the scarcity of papers that have quantified FODMAPs in breads, different concentrations of fructans (0.1-1.7% of dry weight) have been reported in rye, spelt and wheat breads. Fructose (0.1-2.3% of dry weight), GOS (0.1-0.4% of dw), FOS (0.05-0.15% of dw), sorbitol and mannitol (in trace) were also found (Biesiekierski et al., 2011; Laatikainen et al., 2016; Whelan et al., 2011). Besides that, pseudo-cereals and legumes are used for enriching/fortifying bread with

nutritionally important components (e.g., minerals, vitamins, phenolic compounds). This would increase the dietary intake of FODMAPs (especially GOS), because pseudo-cereals and legumes contain remarkable amounts of raffinose and stachyose (Muir et al., 2009; Curiel et al, 2015).

As stated in the introduction, bread accounts for a major part of daily consumed FODMAPs. Although the individual concentration of each subgroup of carbohydrates that comprises the FODMAPs may be low - except fructans, which are found in relatively high concentrations - consider the total content of all the subgroups of FODMAPs is more important than the individual levels of each subgroup, since the main effects of its intake can be summative. The fact that bread is one of the most important carbohydrate sources of the daily diet makes its quantity of FODMAPs relevant to IBS-sufferers.

Baker' yeast-leavened bread is typically obtained upon short fermentation (0.5-3 h), which causes a relatively limited hydrolysis of cereal components, including proteins and FODMAPs. On the opposite, sourdough biotechnology requires longer fermentation time. Sourdough is a complex microbial ecosystem, usually dominated by heterofermentative lactobacilli (e.g., *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus rossiae*, *Lactobacillus reuteri*, *Lactobacillus sanfranciscensis*) and yeasts (e.g., *S. cerevisiae*, *Kazachstania exigua*, *Kazachstania humilis*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *Pichia kudriavzevii*). Microbial ecology of this ecosystem has been widely described in other reviews (De Vuyst et al., 2014; Gobbetti et al., 2016).

Sourdough biotechnology may be exploited to improve flavor and texture, extend shelf-life and enhance nutritional and functional quality of leavened baked goods (Gobbetti et al., 2014). Most of these features are due to metabolic activities of LAB, especially on carbohydrates and proteins. During sourdough fermentation, the metabolism of carbohydrates depends on the available substrates, microbial and endogenous (flour) enzymes and interactions between microbial populations (e.g., competition, proto-cooperation). In detail, during typical (wheat, rye) sourdough fermentation, flour α-amylase hydrolyzes starch to maltodextrins, which are then converted by flour β-amylase into maltose, the most abundant fermentable carbohydrate in dough. At dough stage, microbial invertase rapidly cleaves flour sucrose into glucose and fructose (Gänzle, 2014). Glucose is used as energy source, whereas fructose may be reduced by heterofermentative LAB to

mannitol. Through reduction of fructose to mannitol, these bacteria convert acetyl-phosphate to acetate (instead of ethanol), thus gaining an extra mole of ATP (Gobbetti et al., 2005). Overall, all the fermentable carbohydrates (sucrose, maltose, glucose, fructose) are quickly depleted during the first hours of fermentation, whereas carbohydrates with a higher degree of polymerization (such as fructans) are used later. This leads to hypothesize that long fermentation, such as that typically relying on sourdough, can provide a more pronounced degradation of FODMAPs. Contrarily to starch and fermentable carbohydrates, the mechanisms behind FODMAPs degradation have received less attention.

Among FODMAPs, fructans in wheat could be degraded upon sourdough fermentation. Indeed, few sourdough LAB, such as *Lactobacillus amylovorus* and *Lactobacillus crispatus*, are able to metabolize fructans (Loponen and Gänzle, 2018). Muller & Lier (1994) described that fructans are converted to fructose and sucrose, which are further metabolized into lactic acid by homofermentative LAB or into lactic acid, acetic acid, ethanol and CO_2 by heterofermentative LAB. In addition, reduction of fructose to mannitol, another FODMAP, frequently occurs in sourdough fermented by heterofermentative LAB (Gänzle, 2015). An extracellular, cell wall-associated β-fructosidase has been reported in strains of *Lactobacillus paracasei* as responsible for the degradation of fructans, leading to extracellular accumulation of sucrose and fructose (Goh et al., 2007; Makras et al., 2005). *L. plantarum*, *L. fermentum*, *L. brevis*, and *Lactobacillus buchneri*, isolated from vegetables, produce α-galactosidase, acting on GOS (Silvestroni et al., 2002). In addition, gene coding for levansucrase was expressed by *Lactobacillus reuteri* LTH5448, isolated from sourdough, more than 100 fold in presence of raffinose (Teixeira et al., 2012). This strain, also possessing α-galactosidase activity, was used to ferment (37 °C, 24 h) faba bean flour, which was added to gluten-free bread dough containing, among other ingredients, baker's yeast and sorghum sourdough (previously fermented by the same strain). After 2 h of fermentation, GOS had been totally degraded. However, partial degradation of GOS to melibiose, manninotriose or manninontetraose was also observed when unfermented bean flour was used as ingredient, indicating that extracellular levansucrase rapidly acts on GOS, whereas subsequent intracellular α-galactosidase activity proceeds slower. Comparable partial degradation of GOS was also observed in the control gluten-free dough, wherein *L. reuteri* LTH5448 had not been used (Teixeira et al.,

2012). This could be explained by the action of yeast's invertase, which has a catalytic mechanism similar to levansucrase (Lammens et al., 2009). The invertase produced by *S. cerevisiae* is also able to degrade (partially) fructans to glucose and fructose. Indeed, during wheat whole meal bread-making, *S. cerevisiae* degraded almost 80% of the fructans initially present (Verspreet et al., 2013). Overall, yeasts may reduce the level of various FODMAPs during bread-making. For instance, Ziegler et al. (2016) showed that relatively long (4.5 h) baker's yeast fermentation results quite effective for reducing up to to 90% the dough levels of fructans, raffinose, and excess of fructose, in five *Triticum* species. Struyf et al. (2017) developed an efficient yeast-based strategy, using an inulinase-secreting *Kluyveromyces marxianus* strain to reduce by more than 90% fructans levels in bread, upon 2.5 hours of dough fermentation.

Based on the above-mentioned enzymatic activities, some sourdough lactobacilli (*L. plantarum*, *L. fermentum* and *L. brevis*) could ferment GOS, FOS and fructans (Kunová et al., 2011; Pan et al., 2009; Saulnier et al., 2007). In turn, when lactobacilli hydrolyze fructans, polyols and excess fructose are generated. Strains of *L. plantarum* and *Lactobacillus curvatus* isolated from rye sourdough were able to ferment fructose, mannitol and sorbitol (Bartkiene et al., 2017). Wheat germ subjected to long fermentation with autochthonous strains of *L. plantarum* and *L. rossiae* showed 87% and 45% decrease of fructose and raffinose, respectively, compared to the initial concentrations (Rizzello et al., 2010). Similarly, the combined use of *L. plantarum* and *L. brevis* to ferment (at 30 °C, for 24 h) pulses flour resulted in a decreased concentration of raffinose (of up to ca. 64%), with respect to a control dough (obtained without bacterial inoculum) (Curiel et al., 2015).

Costabile et al. (2014) investigated the effects of breads, which differed in terms of fermentation time and biological leavening agent, on the colonic microbiota. *In vitro* batch culture experiments were run using feces from three subjects suffering from IBS and three healthy subjects. A significant increase in intestinal bifidobacterial populations, cultured *in vitro*, occurred after 8 hours of fermentation with pre-digested sourdough breads. The same effect was not observed for breads produced with commercial yeast. Bifidobacteria do not produce gas and have been shown to benefit health of IBS patients (Clarke et al., 2012). δ-Proteobacteria and most Gemmatimonadetes species decreased in both IBS and healthy subjects upon 24 hours-long exposure to sourdough

bread. In addition, in IBS subjects the latter produced significantly lower cumulative gas after 15 h of exposure, compared to baker's yeast breads. Overall this study suggests that sourdough bread may be beneficial for patients suffering from IBS, by influencing composition and metabolic profile of the human intestinal microbiota (Costabile et al., 2014). Another study has shown that low-FODMAPs rye breads, with approximately 30% reduction of mannitol and fructans, caused less gastrointestinal symptoms, as abdominal pain, flatulence, stomach rum- and intestinal cramps (Laatikainen et al., 2016). A third study conducted by Pirkola et al. (2018) demonstrated that low-FODMAP rye bread leads to reduced colonic fermentation and flatulence severity when compared with regular rye bread consumption.

5. FUTURE ASPECTS

Future researches focusing on FODMAPs dietary contribution of bread will have to fill several knowledge gaps. First, information about FODMAPs content in food is limited, making difficult to understand their physiological importance (Giorgio, Volta, & Gibson, 2015; Muir et al., 2009). With special regard to bread, FODMAPs concentration depends on intrinsic characteristics (e.g., cereal variety, growing locations, climate, season), ingredients of bread recipes and processing methods. This gap is hard to be filled also because different methodologies of extraction and detection may lead to different concentrations. When detected through chromatography, some FODMAPs co-elute, obliging to perform a second chromatographic step with two mobile phases. In addition, some FODMAPs are present at concentrations very close to the limit of chromatographic detection, which may cause errors in quantification (Paramithiotis et al., 2007). Finally, detection of some FODMAPs (e.g., fructans) requires a combination of chromatography and enzymatic hydrolysis (Biesiekierski, et al., 2011; Muir et al., 2009). Overall the finding of well-standardized methods of FODMAPs detection would be a key-point for food researchers, industries and consumers. Besides knowing FODMAPs concentrations, it is of utmost importance to elucidate the degradation pathways of the different FODMAPs subgroups and how they affect intestinal microbiota. This would help to evaluate the different symptomatic responses of subjects and to guide researchers in designing foods targeted for any specific group of consumers (Tuck et al., 2014).

As discussed in the previous paragraph, LAB and yeasts may degrade FODMAPs. Compared to baker's yeast, sourdough shows a great potential for driving FODMAPs concentrations of bread (and, overall, leavened baked goods), because it often includes both microbial populations. Therefore, future research should focus on how to combine enzyme activities of LAB and yeasts to reach the desired goal. In this context, given that some FODMAPs degrading enzymes are rather widespread in LAB, one pillar is the selection of microbial strains possessing such enzymes and their use as sourdough starters.

Finally, the effect of FODMAPs degradation on bread quality should be evaluated. For instance, sucrose and fructose, generated upon partial hydrolysis of fructans, may be further used by bacteria to synthesize exopolysaccharides. These compounds protect bacteria against various environmental stresses, improve texture and delay bread staling (Dertli et al., 2016; Ketabi et al., 2008). In addition some exopolysaccharides have prebiotic activity (Ketabi et al., 2008; Torrieri et al., 2014). On the other hand, eventually, the EPS produced can be a fructose-composed EPS and the degradation of this EPS during the dough preparation may again result in an increase in the fructose concentration, which is not favorable for IBS-sufferers. Besides that, some LAB reduce fructose to mannitol, which would reduce the level of fructose but increase the other FODMAP. In this sense, the effects of sourdough fermentation on the content of FODMAPs will depend on the fermentation conditions and on which LAB species will be applied, offering a rather uncovered field to be clarified. As well as the possible increase in mannitol levels may affect the health of IBS-sufferers requires further studies.

Filling these knowledge gaps, it will be possible to modulate FODMAPs type and concentration in bread, allowing to reach two goals: (i) limiting the intake of FODMAPs in IBS patients to avoid undesirable gastrointestinal symptoms; (ii) attempting to increase their intake in healthy subjects.

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CHAPTER III – SOURDOUGH MICROBIAL DYNAMICS REVEALED BY METAGENOMIC ANALYSIS IN BRAZIL

SOURDOUGH MICROBIAL DYNAMICS REVEALED BY METAGENOMIC ANALYSIS IN BRAZIL

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Abstract This study dealt with the influence of the temperature on the bacterial dynamics of two spontaneously fermented wheat sourdoughs, propagated at 21 ± 1 ºC (SD1) and 30 ± 1 ºC (SD2), during nine backslopping steps. *Proteobacteria* was the only phylum found in flour. *Escherichia hermannii* was predominant, followed by *Kosakonia cowanii*, besides species belonging to the genera *Pantoea* and *Pseudomonas*. After one step of propagation, *Clostridium* and *Bacillus cereus* group became predominant. *Lactobacillus curvatus* was found at low relative abundance. For the second backslopping step, *Clostridium* was flanked by *L. curvatus* and *Lactobacillus farciminis*. From BS4 onward, lactic acid bacteria (LAB) became predominant. *L. farciminis* overcame *L. curvatus* and remained dominant until the end of propagations for both sourdoughs. At 21 ºC ± 1ºC, *Bacillus*, *Clostridium*, *Pseudomonas*, and *Enterobacteriaceae* were gradually inhibited. At the end of propagation, SD1 harbored only LAB. Otherwise, the temperature of 30 ºC ± 1ºC favored the persistence of atypical bacteria in SD2, as *Pseudomonas* and *Enterobacteriaceae*. Therefore, the temperature of 21 ºC ± 1ºC was more suitable for sourdough propagation in Brazil. This study enhanced the knowledge of temperature's influence on microbial assembly and contributed to the elucidation of sourdough microbial communities in Brazil

Keywords: fermentation, bacterial diversity, high-throughput sequencing, enterobacteria, lactic acid bacteria.

1. INTRODUCTION

Sourdough results from the fermentation of cereal flour and water, by a microbial consortium, composed mainly by lactic acid bacteria (LAB) and yeasts. The sourdough fermentation is known to contribute in several ways to the enhanced nutritional, sensorial and technological qualities of leavened bakery products, due mostly to the metabolic activity of its microbial community (De Vuyst et al., 2014; Gobbetti et al., 2018; Minervini et al., 2014). The dough is a nutrient-rich ecosystem for microbial growth. More than 80 LAB and 20 yeast species have been isolated around the world from mature sourdoughs. *Lactobacillus*, *Leuconostoc*, *Weissella*, *Saccharomyces,* and *Kazachstania* are the most frequent genera described (Gänzle and Ripari, 2016; Gobbetti et al., 2016; Van Kerrebroeck et al., 2017).

Traditional sourdoughs require continuous steps of fermentation (backslopping). The first dough prepared using flour and water is spontaneously fermented at room temperature. Posteriorly, this fermented dough will be used as inoculum for fermenting a new dough in the subsequent step. This procedure is repeated five to ten times (Minervini et al., 2014; Siepmann et al., 2018). The sourdough microbial consortia evolves from the first fermentation and through the backslopping steps, resulting in both successions of microbial populations and alteration of metabolic patterns until the microbiota becomes stable. This dynamics is affected by numerous endogenous and exogenous factors, such as flour type and origin, environmental microbiota, process parameters (*e.g.* temperature, redox potential, refreshment time, number of propagation steps) and interactions between the microbial consortium (De Vuyst et al., 2014; Gobbetti et al., 2016; Minervini et al., 2014; Van Der Meulen et al., 2007; Vogelmann and Hertel, 2011a).

The positive effects of LAB on sourdough bread quality has been demonstrated in many studies (Arendt et al., 2007; Corsetti and Settanni, 2007; Gänzle and Ripari, 2016; Gänzle et al., 2008, 2007; Gobbetti et al., 2014; Katina et al., 2005; Pétel et al., 2017; Poutanen et al., 2009; Torrieri et al., 2014). More attention has now been paid to the nutritional properties of sourdough breads, in order to investigate the possibilities of fermentation as a tool to prevent and treat diseases. For instance, nowadays it is well know the bacteria ability to decrease the gluten immunogenicity through enzymatic degradation by microbial proteases (Curiel et al., 2013; De Angelis et al., 2010; Heredia-Sandoval et al.,

2016). The use of sourdough in bakery production has potentiality to reduce the Irritable Bowel Syndrome (IBS) and the Non-Celiac Gluten Sensitivity (NCGS) symptoms (Menezes et al., 2018; Muir et al., 2019). The degradation of fructan and other FODMAPs implicated in triggering the symptoms of IBS and NCGS was recently demonstrated during sourdough fermentation reported in our previous study (Menezes et al., 2019).

Sourdough proved to be an inexhaustible source of microbial species in the countries where it has been studied. Although broadly investigated in European countries, USA, and most recently in Asian countries (Corsetti and Settanni, 2007; De Vuyst et al., 2014; Gobbetti, 1998; Lattanzi et al., 2013; Lhomme et al., 2015; Liu et al., 2016a; Ventimiglia et al., 2015), the microbial diversity of sourdoughs has not yet been characterized in Brazil. The geographic origin and the propagation temperature have been shown to exert a strong influence on LAB diversity (Pontonio et al., 2015; Scheirlinck et al., 2007). Uncovering the correlation between microbial species and their role in a specific ecosystem remains one of the main objectives of microbial ecology (Morales and Holben, 2011).

Regarding sourdough, knowledge about the fermenting microbial consortia contributes to the understanding of its influence on the bread quality. The interdependence between process parameters and microbial dynamics is a field of interest for the bakery industry, since standardization of bread quality is dependent on the microbial community (Gobbetti et al., 2016; Menezes et al., 2019). Thus, this study aimed at unraveling how the temperature affects the dynamics of the microbial assembly during the propagation of sourdoughs in Brazil. With the aim to lead to the standardization of sourdough fermentation performance, allowing its safe and controllable use, this research is a step forward the elucidation of the microbial succession and the factors that affect it.

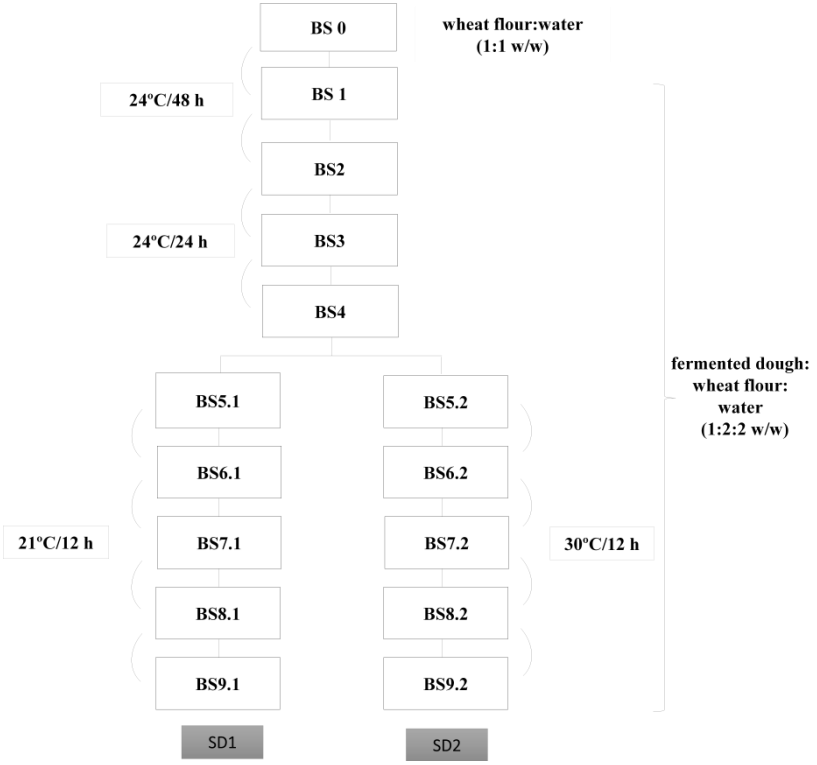
2. MATERIALS AND METHODS

2.1 SOURDOUGH PROPAGATION

Sourdoughs were made through traditional protocol - sourdough type I, mixing organic wheat flour (Paullinia company, Marechal Cândido Rondon, Paraná, Brazil) and mineral water [1:1 (w/w)] with a resulting dough yield [(dough mass/flour mass) × 100] of 200 (Figure

1). The first fermentation was carried out at 24 °C for 48 h (backslopping one - BS1). Successively, eight backslopping steps (BS2 to BS9) were carried out. In each one, a portion of the previously fermented dough (FD) was harvested and used as an inoculum for the subsequent step, mixed with wheat flour and water [FD:water:wheat flour (1:2:2 w/w)]. The mixture was incubated at 24 °C for 48 h at BS2 and 24 h at BS3 and BS4. Thereafter, the FD was fractionated in two portions; the first one was incubated at 21 ± 1 ºC (SD1) and the second one at 30 ± 1 ºC (SD2). Finally, the BS5 to BS9 were carried at 12 h intervals. The fermentations were carried out in a Biochemical Oxygen Demand (BOD) Refrigerated Incubator (MA 403 Marconi, Piracicaba, São Paulo, Brazil) with temperature control.

Figure 1.Set up of fermentations



2.2 MICROBIAL ENUMERATION AND BACTERIAL ISOLATION

Ten grams of the flour and BS samples were homogenized by adding 90 ml 0.1% ($w.v^{-1}$) of sterile peptone solution using a vortex. A 10-fold dilution series were made and plated in the culture media presented in table 1. The results were expressed as log $CFU.g^{-1}$. A total of 100 colonies with different morphologies (shape, size, and colors) were randomly picked from the plates, cultivated in respective broth media and re-streaked onto the same agar medium to check the purity. Posteriorly, the isolates were lyophilized (LT1000, Terroni, São Carlos, Brazil) for 24 h (90 µHg of vacuum), before fingerprinting and identification.

Table 1. Culture media used for isolation.

Media	Composition	Incubation conditions	Reference
mMRS	MRS agar modified by addition of maltose 1%	37 ºC/48 h anaerobic	Lhomme et al. (2015)
M17	[1]	30 ºC/48 h	Li; Li; Bian (2016)
MYP	Mannitol 2.5%, yeast extract 0.5%, peptone 0.3%, agar 2%	30 ºC/48 h	Nguyen et al. (2015)
SDAM	Sourdough 3%, agar 2%	30 ºC/48 h	[2]
WFAM	Wheat flour 3%, agar 2%	30 ºC/48 h	[2]

[1]Unchanged commercial composition. [2] Formulated by the authors.

2.3 DNA EXTRACTION

The isolates were cultured in the respective origin medium and incubated overnight at the temperature indicated in table 1. The cultures (1 mL) were centrifuged (10,000 *g*, 10 min) and the DNA was extracted using the Genomic Wizard DNA Purification Kit (Promega Corp., Madison, WI, USA) and stored at -20 ºC until use. Total genomic DNA of the flour, doughs after the backslopping steps and mature sourdoughs was extracted directly from 1 g of the samples using the DNeasy Blood & Tissue kit (Qiagen, Venlo, Netherlands). DNA was eluted into DNase- and RNase-free water and concentration and purity were determined using a NanoDrop spectrophotometer (model 2000,

ThermoFisher Scientific Inc, Waltham, Massachusetts, EUA). DNA was diluted up to 50 ng.μL^{-1} and stored at -20 ºC until use.

2.4 METAGENOMIC ANALYSIS

The total DNA extracted from the flour and sourdough samples was used as template for 16S metagenomic analysis, which was performed by Neoprospecta Microbiome Technologies (Florianopolis, Brazil) using the Illumina MiSeq platform (Illumina Inc., San Diego, California). Library preparation was performed using Neoprospecta´s NGS Procotol (CHRISTOFF et al., 2017). Briefly, the V3-V4 hypervariable region of the 16S rRNA gene was amplified with primers 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) (WANG et al., 2009; CAPORASO et al., 2011). The PCR reaction was carried out in triplicates using Platinum Taq Polymerase (Invitrogen, USA) with the following conditions: 95 °C for 5 min, 25 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s and a final extension of 72 °C for 2 min. Library preparation (attachment of TruSeq adapters, purification with AMPureXP beads and qPCR quantification) was performed using Illumina 16S Library Preparation Protocol (Illumina Technical Note 15044223 Rev. B). Sequencing was performed using MiSeq Reagent Kit v3 with 2x300 bp paired-end reactions.

2.5 BIOINFORMATICS

Sequencing data for each sample was processed on Quantitative Insights into Microbial Ecology (Qiime) software package (CAPORASO et al., 2010). Initially, the sequencing output was analyzed by a read quality filter, which removed reads with an average Phred score < 20 followed by a clustering of 100% identical reads. In order to remove putative chimeric sequences, clusters with less than 5 reads were excluded from further analysis. The remaining good-quality sequences were further clustered at 97% similarity to define operational taxonomic units (OTU). Classification of OTUs was made by comparing them with a custom 16S rRNA database (NEORefDB, Neoprospecta Microbiome Technologies, Brazil). Sequences were taxonomically assigned with at least 99% identity in the reference database. In order to evaluate the microbial community shifts among

samples, OTUs were summed up into the same genera and the relative abundance of each genus was compared with a heatmap on Qiime.

2.6 LENGTH HETEROGENEITY-PCR (LH-PCR)

Total DNA extract from the isolates and SD samples were analyzed following the LH-PCR amplification as described by Savo Sardaro et al. (2018) to better understand the bacterial succession ecology through the backslopping steps. Domain A of the variable regions of the 16S rRNA gene from extracted DNA was amplified. The forward primer, 63F (5'-CAGGCCTAACACATGCAAGTC-3') (Marchesi et al., 1998) was 5' end labeled with the phosphoramidite dye 6-FAM and the reverse primers used were 355R (5'-GCT GCC TCC CGT AGG AGT-3') (Applied Biosystems Inc., Foster City, USA). In each PCR amplification, 1 µl of extracted DNA was added to 19 µl of the amplification mixture, resulting in a final concentration of 1X Taq Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 mM of each primer, and 1U of Taq DNA polymerase (Promega), in a final reaction volume of 20 µl. PCR conditions were as follows: an initial denaturation at 95 °C for 5 min, 25 cycles of denaturation at 95 °C for 30 s; different annealing temperature were used (59 °C for SD and 63 °C and 65 °C bacteria strain) for 30s; elongation at 72 °C for 1 min 30 s, and a final extension step at 72°C for 7 min. PCR products amplified were diluted 15 time fold for subsequent fragment analysis as described below.

Capillary electrophoresis (ABI Prism 310, Applied Biosystems, Foster City, USA) were performed according to Bottari et al. (2010). Each peak on the electropherogram profile corresponds to an amplicon with specific length (in base pairs, bp). The obtained lengths from the strains were used as a reference to identify the species corresponding to single peaks in the LH-PCR profile of the SD bacterial population.

2.7 REPETITIVE ELEMENT PALINDROMIC-PCR (REP-PCR)

The rep-PCR was performed using DNA extracted from the 100 isolated strains. PCR reactions were performed according to Perin et al. (2017), using a single primer (GTG)5 (5'-GTGGTGGTGGTGGTG-3'). The PCR reactions contained 10 mL of Go Taq Master Mix 2x (Promega, Madison, Wisconsin, EUA), 50 pMol of the primer, 2 mL of DNA (50 ng/mL) and ultra-pure PCR water (Promega) to a final volume

of 20 mL. The PCR conditions were: 95 ºC for 5 min, 30 cycles at 95 ºC for 30 s; 40 ºC for 45 s; 65 ºC for 8 min; and final extension at 65 ºC for 16 min. The PCR products were electrophoresed on agarose gels (2% w/v) in tris/borate/EDTA buffer (TBE) at constant voltage (95 V) for 3 h. A 1 kb DNA ladder (Sigma-Aldrich, St. Louis, Missouri, EUA) was used as a molecular size marker. Fingerprints were compared by cluster analysis using BioNumerics 6.6 (Applied Maths, Sint-MartensLatem, Belgium). Similarities between the strains profiles were calculated using the Dice correlation coefficient and dendrograms constructed by cluster analysis (unweighted pair group method with arithmetic mean, UPGMA).

2.8 BACTERIAL IDENTIFICATION

Based on rep-PCR profiles and similarities, 41 isolates were selected and subsequently identified by 16S rRNA sequencing using the primers forward 46F (GCYTAACACATGCAAGTCGA) and reverse 536R (GTATTACCGCGGCTGCTGG) (Kaplan and Kitts, 2004). The PCR reactions consisted of 10 mL of Go Taq Master Mix 2x (Promega), 10 pMol of each pair of primers, 1 mL of DNA (50 ng/mL) and ultra-pure PCR water (Promega) to a final volume of 20 mL. DNA amplification and sequencing were performed according to Perin et al. (2017), and each sequence obtained was checked manually and searched for sequence homology using the basic local alignment search tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

2.9 STATICAL ANALYSIS

The values of bacterial enumeration in each culture media were subjected to one-way ANOVA; pair-comparison of treatment means was obtained by Tukey's procedure at $p < 0.05$, using the statistical software *Statistica* 11.0 (StatSoft Inc., Tulsa, USA). The effect of temperature incubation on SD1 and SD2 samples was evaluated independently by a Pearson correlation test. In this analysis, the absolute abundance of *Lactobacillus* and *Lactococcus* (namely “Lacto” group) in each SD was tested for correlation against genera *Bacillus*, *Pseudomonas*, *Clostridium*, *Escherichia*, *Enterococcus* and *Enterobacter* in that sample. A significant effect was considered on $p < 0.05$.

3. RESULTS

The presumptive LAB counts in mMRS for flour were 3.0 ± 0.1 log CFU.g^{-1} (Table 2). After BS1, cell density of presumptive LAB in mMRS increased significantly to 5.7 ± 0.1 log CFU.g^{-1}. The counts reached 7.1 ± 0.0 log CFU.g^{-1} for BS2. For SD1, for BS5, the cell density reached 7.5 ± 0.1 log CFU.g^{-1} and stayed almost constant during the subsequent propagations, despite a slight fluctuation in BS8. For SD2, from BS5 onward there was no statistical difference between counts in mMRS. In general, counts of viable microorganisms were lower in the other culture media and evolved more slowly, reaching above 7.0 log CFU.g^{-1} only from BS6 for Wheat Flour Agar Medium (WFAM) and Sourdough Agar Medium (SDAM).

Clusterization by LH-PCR and REP-PCR (Table 3) of the 100 randomly selected colonies were used to classify and select those that would belong to different species and would be sequenced. Only a small part of the sourdough population could be recovered by the culture-dependent method, a quite homogeneous population, with 11 biotypes. Each biotype was taxonomically characterized through 16S rRNA gene partial sequencing. The LAB isolated belonged to the species *Lactobacillus farciminis*, *Lactobacillus brevis*, *Lactococcus lactis*, *Leuconostoc citreum* (two biotypes), *Enterobacter hormaechei/cloacae*, *Enterococcus gilvus*, *Enterococcus hirae*, *Enterococcus durans*, *Enterococcus faecium* and *Enterococcus faecalis*. *Ec. faecium* and *L. brevis* were the most dominant species. While some species of *Enterococcus* were present variably, *L. brevis* was found from BS2 and persisted until the final propagation step. *Lc. lactis* was isolated from BS2 to BS4 and persisted only for SD2, until BS7. *Eb. hormaechei/cloacae* was recovered from BS1 and BS6. The first biotype of *Ln. citreum* was recovered from BS5 of SD2; the second one was isolated from BS7 and BS8 from SD2 and SD1, respectively. *L. farciminis* was isolated only in BS8 and BS9, in both SD.

Table 2. Cell density of bacteria (log CFU.g^{-1}) for backslopping steps of sourdough propagation.

	mMRS		M17		WFAM		SDAM		MYP	
Flour	3.0±0.1^{A,a}		3.0±0.0^{A,a}		3.0±0.2^{A,a}		3.7±0.1^{A,b}		3.8±0.2^{A,b}	
BS0	3.0±0.1^{A,a}		4.5±0.3^{B,b}		4.3±0.2^{B,b}		4.5±0.0^{B,b}		4.2±0.1^{B,b}	
BS1	5.7±0.1^{B,c}		5.5±0.4^{C,c}		3.5±0.1^{A,B,a}		4.9±0.1^{B,C,b}		6.0±0.0^{D,c}	
BS2	7.1±0.2^{C,c}		5.9±0.2^{C,D,b}		4.2±0.2^{B,a}		5.5±0.0^{C,b}		5.9±0.0^{D,b}	
BS3	7.5±0.0^{D,c}		5.0±0.2^{B,C,a,b}		4.4±0.3^{B,a}		5.5±0.1^{C,b}		5.3±0.3^{C,a,b}	
BS4	7.1±0.0^{C,c}		4.9±0.1^{B,C,a}		6.7±0.1^{C,b}		5.0±0.1^{B,C,a}		6.4±0.0^{E,b}	
	SD1	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**
BS5	7.5±0.1^{D,e}	7.5±0.1^{D,e}	5.4±0.4^{C,a,b}	6.3±0.1^{D,c}	7.1±0.1^{D,d}	6.5±0.1^{C,c}	4.9±0.1^{B,C,a}	5.6±0.0^{C,b}	6.5±0.2^{E,c}	6.7±0.1^{E,F,c}
BS6	7.4±0.2^{D,c}	7.4±0.2^{D,c}	6.5±0.0^{D,a}	6.5±0.0^{D,a}	6.8±0.0^{C,b}	7.2±0.1^{D,c}	7.2±0.0^{D,c}	7.3±0.1^{D,c}	6.6±0.2^{E,a,b}	7.3±0.2^{F,c}
BS7	7.6±0.1^{D,d,e}	7.6±0.4^{D,d,e}	6.3±0.0^{D,a}	6.5±0.1^{D,a}	7.1±0.0^{D,b}	7.4±0.1^{E,d,e}	7.2±0.1^{D,c,d}	7.3±0.1^{D,c,d}	6.5±0.4^{E,a}	7.3±0.0^{F,c,d}
BS8	7.2±0.1^{C,d}	7.4±0.2^{D,d}	7.0±0.0^{E,c}	6.3±0.2^{D,b,}	7.3±0.1^{D,d}	7.4±0.0^{E,e}	7.1±0.0^{D,c}	7.3±0.0^{D,d}	5.9±0.1^{D,a}	6.6±0.3^{E,b}
BS9	7.5±0.0^{D,e,d}	7.5±0.0^{D,e,d}	5.1±0.0^{B,C,a}	6.9±0.0^{D,E,c}	7.3±0.0^{D,c}	7.4±0.2^{E,e}	7.2±0.1^{D,c}	7.3±0.0^{D,c}	7.0±0.1^{E,F,c}	6.5±0.1^{E,b}

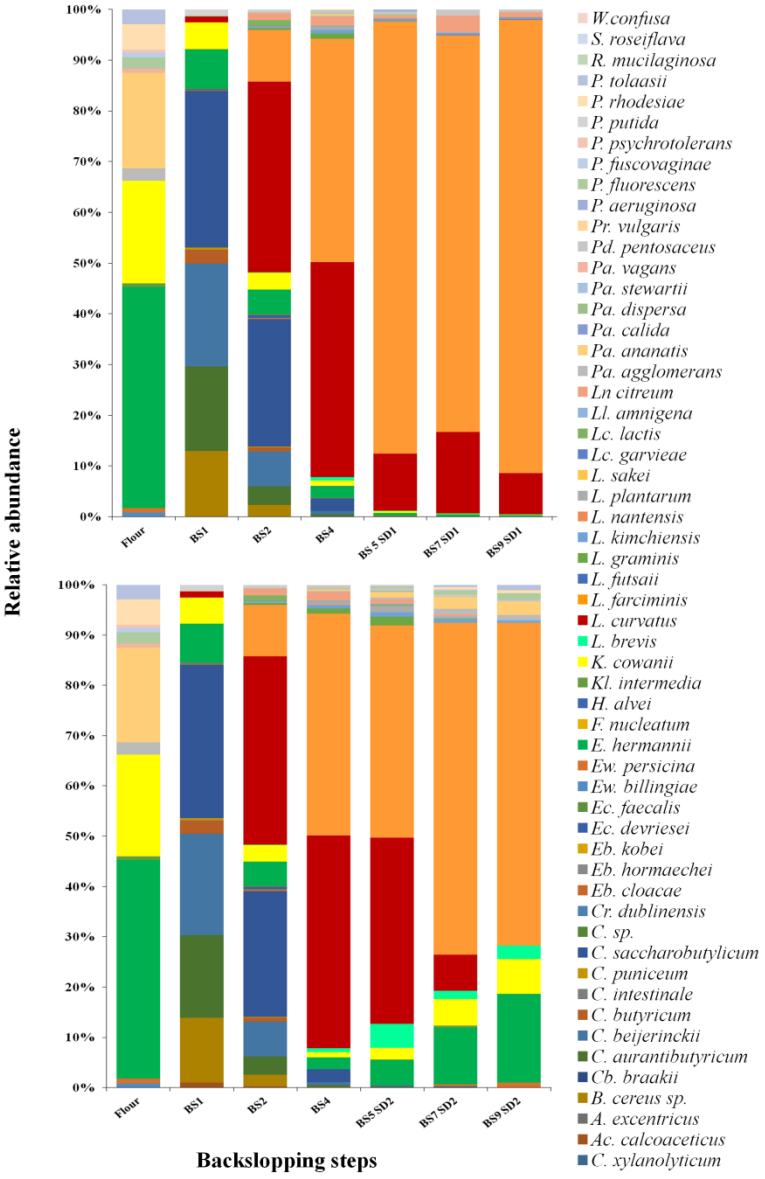
$^{A-B}$ Values in the same column with different superscript letters differ significantly *($p < 0.05$)*
$^{a-b}$ Values in the same row with different superscript letters differ significantly *($p < 0.05$)*

Table 3. Rep-PCR Clusterization and LH-PCR fragment length database of bacteria isolated from sourdough with corresponding identification obtained by 16S rRNA sequencing.

Rep-PCR cluster	Number of isolates	GeneBank closest relative	Accession number	% Match	LH-PCR fragment length (bp)	Step of isolation SD – BS*
I	02	*Ec. durans*	NZ_CP022930.1	99%	330	SD1 – 8 SD2 – 6
II	06	*Ec. faecalis*	NZ_CP008816.1	99%	330	SD1 – 6, 8, 9 SD2 – 6, 8
III	30	*Ec. faecium*	NC_017960.1	100%	330	1, 2, 3, 4, SD1 – 5, 7, 8, 9 SD2 – 5, 6, 8, 9
IV	02	*Ec. gilvus*	NZ_ASWH01000001.1	98%	330	3, SD2 – 5
V	08	*Ec. hirae*	NC_0180801.1	100%	330	3, 4, SD1– 9 SD2 – 6, 8
VI	05	*Eb. hormaechei/ cloacae*	NZ_CP017179.1 NZ_CP008823.1	99%	310	1 SD1 and 2 – 6
VII	26	*Lb. brevis*	NZ_CP024635.1	100%	336	2,3 SD1 –5, 6, 7, 8, 9 SD2 – 6, 7, 9
VIII	04	*Lb. farciminis*	NZ_GL575020.1	99%	336	SD1– 8, 9 SD2 – 8, 9
IX	10	*Lc. lactis*	LKLC00000000	100¨%	318	2, 3 SD2 – 5, 7
X	02	*Ln. citreum*	CP024929.1	99%	317	SD2 – 5
XI	02	*Ln. citreum*	CP024929.1	99%	317	SD1 – 8

*1 to 4 – Backslopping steps were the dough had not yet been portioned. From the backslopping step 5 (BS5), the dough was divided in SD1 and SD2, therefore, the isolate comes from SD1 and/or SD2, and belongs to one of the subsequent propagation steps (BS5 to BS9).

Figure 2. Relative abundance (%) of bacterial species during sourdough propagation.



DNA extracted from the flour and sourdough samples was used as template for 16S metagenomic analysis to describe the bacterial diversity (Figure 2). The flour microbial consortium was composed of thirteen different species belonging to *Proteobacteria* phylum. *Escherichia hermannii* (relative abundance of 43.56%) was predominant, followed by *Kosakonia cowanii* (20.21%), and *Pantoea ananatis* (18.85%). *Pseudomonas rhodesiae* (5.10%), *Pseudomonas tolaasii* (2.90%), *Pantoea agglomerans* (2.42%), and *Pseudomonas fluorescens* (2.24%) were also present. After the BS1, twenty-three species were found. *Firmicutes* - *Clostridium saccharobutylicum* (29.62%), *Clostridium beijerinckii* (19.55%), *Clostridium aurantibutyricum* (15.96%) and *Bacillus cereus* group (12.44%) became predominant. *E. hermannii* (7.45%), and *K. cowanii* remained representative (5.02%). *Lactobacillus curvatus* (1.17%), *Lc. lactis* (0.07%), *Ln. citreum* (0.02%) and *Pediococcus pentosaceus* (0.02%) were found, however with low relative abundance. *Pseudomonas* corresponded to 1.19%, *Enterococcus* and *Enterobacter* 0.25%. From BS2, the genus *Clostridium* was flanked by LAB. The dough was dominated by *L. curvatus* (37.46%), *C. saccharobutylicum* (25.07%), and *L. farciminis* (10.21%). *E. hermannii* (4.73%) and *K. cowanii* (3.33%) were still present. Other seven LAB species were found – *Lc. lactis* (1.11%), *Lactobacillus graminis* (0.33%), *Lactobacillus kimchiensis* (0.18%), *Lactobacillus plantarum* (0.16%), *Lactococcus garvieae* (0.12%), *L. brevis* (0.09%) and *Lactobacillus sakei* (0.02%) – as well as *Enterococcus*, *Enterobacter*, and *Pseudomonas* (0.82%, 0.25% and 0.3%, respectively).

From BS4, twenty-nine species were found. The dough was dominated by LAB. The relative abundance of *L. curvatus (*42.36%) and *L. farciminis* (44.07%) were higher compared to earlier steps. *C. saccharobutylicum* (2.51%), *E. hermannii* (2.31%) and *K. cowanii* (1.04%) were still found, but at lower relative abundance than in the previous BS. *Pseudomonas* and *Pantoea* corresponded to 0.16% and 0.59%, respectively. For BS5, SD1 was dominated by *L. farciminis* (85.39%) and *L. curvatus* (11.28%). Among the other seventeen species detected, only *Ln. citreum* (0.55%), *Pd. pentosaceus* and *E. hermannii* (0.54%) were found with relative abundance higher than 0.5%. *L. brevis* (0.10%), *L. graminis* (0.12%), *L. kimchiensis* (0.38%), *Lactobacillus nantensis* (0.08%) were present at low incidence. Among the seven species of the genus *Clostridium* present in BS2, only *C. aurantibutyricum* (0.05%) and *C. beijerinckii* (0.02%) remained. *B. cereus* group, *Enterococcus*, and *Enterobacter* were inhibited. Fifteen

different species were detected at BS7, for SD1. The dough was dominated by *L. farciminis* (78.30%), followed by *L. curvatus* (16.03%), *Ln. citreum* (3.31%) and *Pd. pentosaceus* (1.11%). The same sub-dominant LAB species detected in BS5 were found in BS7 but in slightly lower proportions. The genus *Clostridium* was inhibited. *Eb. cloacae* (0.3%), *E. hermannii* (0.46%), *K. cowanii* (0.14%), *P. fluorescens* (0.06%) and two species of the genus *Pantoea* - *Pantoea vagans* and *Pa. ananatis* (both with 0.07%) were the *Proteobacteria* found. At BS9, SD1 harbored eleven species. *L. farciminis* (89.39%) and *L. curvatus* (8.13%) were still predominant. No *Bacillus*, *Pseudomonas*, *Enterococcus*, and *Enterobacteriaceae* were found. *Ln. citreum* (0.97%), *Pd. pentosaceus* (0.52%), *L. brevis (0.03%)*, *L. futsaii* (0.04%), *L. kimchiensis* (0.4%), *L. nantensis* (0.1%) were detected at low incidence.

On the other hand, the higher temperature altered the microbial dynamics for SD2. For the BS5, twenty-one species were found. *L. farciminis* (40.34%) and *L. curvatus* (35.31%) co-dominated the dough. Other nine species were found with relative abundance higher than 0.5% - *E. hermannii* (4.73%), *L. brevis* (4.55%), *K. cowaniii* (2.21%), *L. graminis* (1.62%), *Pa. ananatis* (1.04%), *L. kimchiensis* (0.88%), *L. plantarum* (1.18%), *Ln. citreum* (0.87%), and *Pd. pentosaceus* (0.50%). *L. lactis* were detected at low concentrations at BS5 (0.28%) and BS7 (0.25%). *B. cereus* group, *Enterococcus*, and *Enterobacteriaceae* were inhibited as for SD1. *C. aurantibutyricum* and *C. beijerinckii* were inhibited at BS7 and BS9, respectively. Three species of the genera *Pseudomonas – P. fluorescens, P. rhodesiae,* and *P. tolaasii* – and two of *Pantoea – Pa. agglomerans,* and *Pantoea dispersa* were found at relative abundances below 0.4%. For BS7, *L. farciminis* remained predominant (65.68%). However, the relative abundance of *L. curvatus* was drastically reduced (7.19%), and *E. hermannii* went on to sub-dominate the dough (11.16%). Other twenty-tree species were detected, including *K. cowanii* (5.32%), *Pa. ananatis* (2.33%), *L. brevis* (1.6%), *Pa. agglomerans* (1.06%), *P. fluorescens* (0.84%), *L. kimchiensis* (0.59%), and *Ln. citreum* (0.5%). For BS9, SD2 was dominated by *L. farciminis* (64.06%) and *E. hermannii* (17.58%). Among the sub-dominant LABs detected in previous steps, only *L. brevis* (2.62%), *L. kimchiensis* (0.28%), and *Pd. pentosaceus* (0.28%) were found. The dough harbored thirteen different species. The other species were *K. cowanii* (6.97%), *Pa. ananatis* (2.67%), *P. fluorescens* (1.34%), *P. tolaasii* (1.06%), *Pa. agglomerans* (1.01%), *Erwinia persicina* (1.00%), *P. rhodesiae* (0.61%), and *Lelliottia amnigena* (0.28%).

The distribution of each genus during the BS was shown in Figure 3. A total of 22 genera were found for SD1 (Figure 3A) and 25 for SD2 (Figure 3B), belonging to the phyla *Proteobacteria*, *Firmicutes*, *Fusobacteria* and *Actinobacteria*, of which the first two were the most relevant, grouped in the upper parts of each heat-map. For SD1, the LAB group was distributed from BS2 to BS9. *Enterococcus*, *Clostridium*, *Bacillus*, *Pseudomonas*, and the family of *Enterobacteriaceae* (*Escherichia*, *Kosakonia*, *Erwinia*, *Enterobacter* and *Pantoea*) were more present from BS1 to BS4, having the numbers of sequences reduced as the propagation evolved. For SD2, the highest number of sequences of LAB was observed from BS2 to BS7. *Enterococcus*, *Clostridium* and *Bacillus* were predominantly found from BS2 to BS4, and reduced for the subsequent BS, as well as observed for SD1.

On the other hand, the genus *Pseudomonas* and the group of *Enterobacteriaceae* presented a wide distribution during the propagation, including for the final BS. The Pearson correlation coefficient (Table 4) showed a significant negative correlation between the "Lacto" group *versus* the genera *Bacillus*, *Clostridium*, *Escherichia*, and *Pseudomonas*, for SD1. These genera were found to decrease with the increasing of "Lacto" group relative abundance. For SD2, significant correlation was observed only for the six combined genera, effect that was also observed for SD1. There was no significant relationship between "Lacto" and "Entero" groups. However, the relative low number of *Enterococcus* and *Enterobacter* sequences detected from SD1 to SD4 may explain any unobserved relationship.

Table 4. Pearson coefficients and *p*-values for correlation analysis between the "Lacto" group (*Lactobacillus* and *Lactococcus*) and the Bacillus, *Clostridium*, *Enterobacter*, *Escherichia*, *Enterococcus* and *Pseudomonas* genera individually and combined.

"Lacto" group versus	**SD1 (21°C)**	**SD2 (30°C)**
Bacillus	**-0.898 (p = 0.006)**	-0.379 (p = 0.443)
Clostridium	**-0.795 (p = 0.039)**	-0.126 (p = 0.808)
Enterobacter	-0.45 (p = 0.352)	0.281 (p = 0.579)
Escherichia	**-0.772 (p = 0.029)**	-0.119 (p = 0.796)
Enterococcus	-0.113 (p = 0.827)	0.578 (p = 0.206)
Pseudomonas	**-0.906 (p = 0.002)**	-0.459 (p = 0.285)
All genera combined	**0.99 (p = 0.001)**	**0.99 (p = 0.001)**

Figure 3A. Heatmap showing the relative abundance and distribution of bacterial communities for SD1 propagated at 21 ºC ± 1ºC. The color code takes into account the number of sequences of each genus individually, ranging from black (0.0) assigned to 0 sequences, to white, (1.0) assigned to the total sequences of that genus. Samples were analyzed in duplicates, represented by lowercase letters a and b.

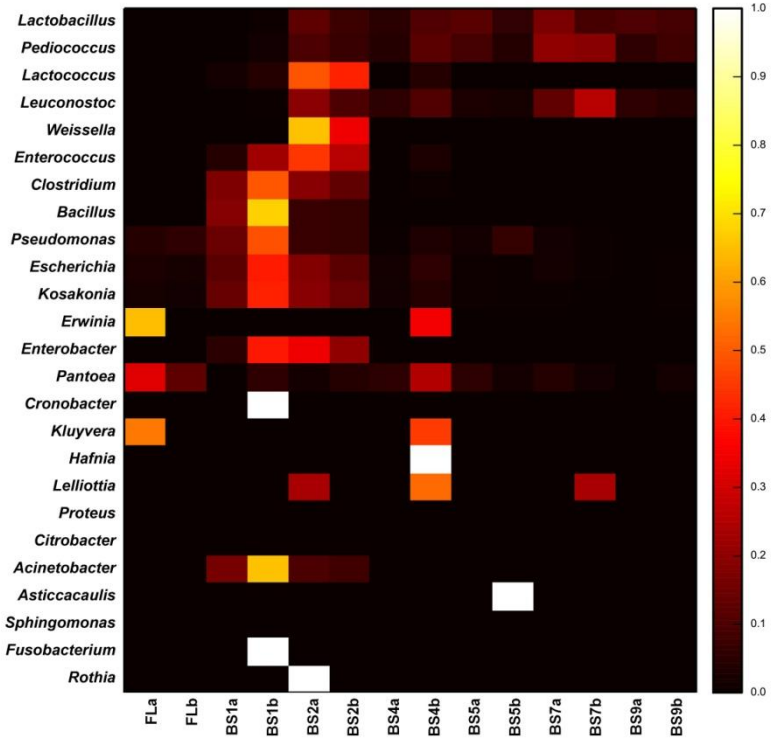
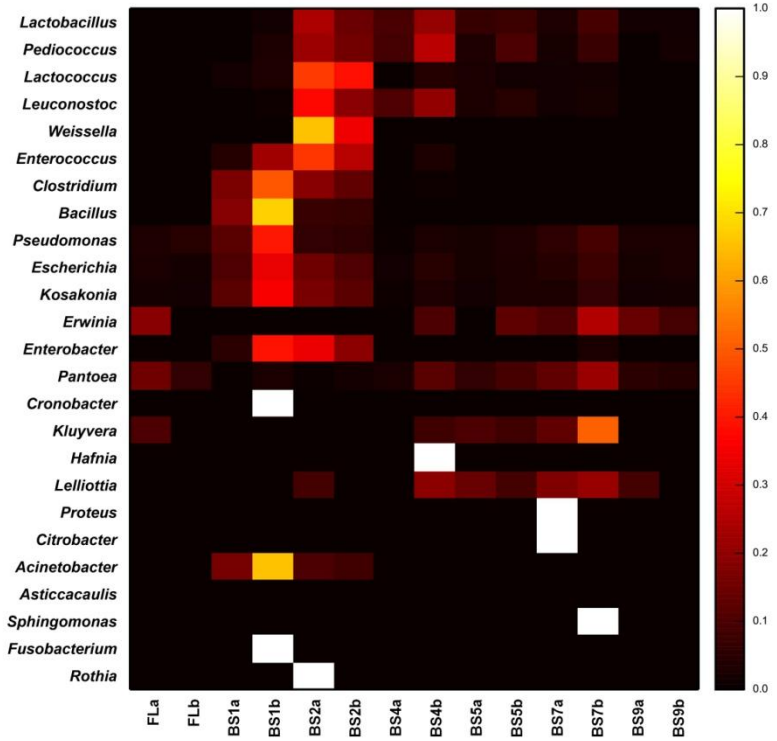


Figure 3B. Heatmap showing the relative abundance and distribution of bacterial communities for SD2 propagated at 30 ºC ± 1ºC. The color code takes into account the number of sequences of each genus individually, ranging from black (0.0) assigned to 0 sequences, to white, (1.0) assigned to the total sequences of that genus. Samples were analyzed in duplicates, represented by lowercase letters a and b.



4. DISCUSSION

Temperature is one of the main parameters that influence the final microbiota composition of sourdoughs (Minervini et al., 2014; Vrancken et al., 2011). This is the first study to our knowledge in which spontaneous sourdough fermentation was followed in Brazil, a country with great climatic diversity. In general, the climate is warm in almost all the territory, with average temperatures above 18 ºC in all months of the year in most states (Brasil, 2002). Two common temperatures in tropical climates were selected for sourdough preparation, and had important implications on the microbial dynamics, especially for the subdominant microflora.

Low counts were found in the flour, and no LAB isolates could be retrieved. The community profiles obtained with pyrosequencing procedure did not detect LAB in the flour, in accordance with its isolates. Before fermentation, low colony counts were found; however, LAB numbers rapidly increased after the BS1. From BS5 onward, the counts were stable for both SD. Other studies (Bessmeltseva et al., 2014, Coda et al., 2018; Ercolini et al., 2013) also described a rapid increase in bacterial counts for the first BS, followed by a relative stabilization. The largest bacterial numbers were found on mMRS, WFAM and SDAM. This finding can be explained by the presence of maltose as source of fermentable carbohydrate in these media. Although some statistically significant differences were detected, it was not possible observing a temperature effect on the cell viable numbers.

Proteobacteria was the only phylum found in flour. Bacteria belonging to this phylum usually composes the microbial community of wheat (Donn et al., 2015; Yin et al., 2017). *Pseudomonas*, *Pantoea*, *Kosakonia*, and *Enterobacter*, commonly prevalent in wheat flour worldwide (Celano et al., 2016; Ercolini et al., 2013), were isolated from Brazilian wheat seeds (Stets et al., 2013). LAB were initially detected from BS1 and BS2. Although flour can drive the microbial diversity of sourdough, along technological parameters of production, the flour microbiota may not be the main source of microorganisms. The house microbiota also affects the composition of LAB and is undoubtedly a critical parameter to establish the sourdough ecosystem (Gobbetti et al., 2016; Minervini et al., 2015). LAB circulate in the bakery environment, and can be found in the hands of bakers, air, and equipment. Indeed, *Lactobacillus* was shown to be the genus with the highest adaptability to bakery environment (Minervini et al., 2015; Scheirlinck et al., 2009).

Notwithstanding *Proteobacteria* is predominant in flour, this phylum is not found often in mature sourdoughs (Ercolini et al., 2013). A succession between *Proteobacteria* and *Firmicutes* occurs gradually from the first propagation to the second one (Weckx et al., 2010b). Just one BS was able to completely turn the microbial community from *Proteobacteria* to mainly *Firmicutes*. Among the species found in the flour, only *E. hermannii* and *K. cowanii* persisted, possibly due to its ability to tolerate the biochemical changes in the matrix. Commonly, *Enterobacteriaceae* grows in the first days of propagation, and survives because of a certain tolerance for acid stress (Ercolini et al., 2013). *B. cereus* is often found in cereals and wheat flour and are well adapted to the bakery environment (Martínez Viedma et al., 2011; Oltuszak-Walczak and Walczak, 2013). Clostridia has quite efficient mechanisms in sugar uptake (Mitchell, 2016). These features, coupled with the semi-anaerobic conditions and the availability of carbohydrates certainly favored the codominance of this groups in BS1.

For BS4 the bacterial profile markedly changed, and *Lactobacilli* completely dominated the SD. There was a marked decrease in pH from BS0 (6.26 ± 0.01) to BS4 (3.79 ± 0.01). The Total titratable acidity (TTA) increased from 1.40 ± 0.13 to 13.85 ± 0.12. Consequently, the highest concentrations of organic acids were found for these BS, as reported in our previous study (MENEZES et al., 2019). Acidification is deeply linked to the assembly of the microbial consortia. The highest concentrations of organic acids coincided with the exponential growth phase of the sourdough communities and are associated with competitiveness between species (De Vuyst et al., 2014). Suppression of *Pseudomonas* (Kiymaci et al., 2018; Nakai and Siebert, 2004), enterobacteria (Skrivanova et al., 2006), *B. cereus* (Soria and Audisio, 2014) and clostridial groups (Schoster et al., 2013; Thylin et al., 1995) is correlated with organic acids synthesis and with a concomitant drop in pH. In turn, LAB are well adapted to the sourdough acid (Corsetti et al., 2007; Corsetti and Settanni, 2007). From the BS2, the dough has become more hostile to *Enterobacteriaceae* and *Clostridium* and more favorable to LAB. When fermentation begins to occur under acidic conditions, evident after the BS2, the growth of non-LAB bacteria is gradually inhibited. Thus, as the number of fermentation steps increases, the LAB becomes more adapted to environmental conditions (Minervini et al., 2014). By definition, LAB are predominant in mature sourdoughs (Gobbetti et al., 2016). LAB can overcome other contaminating microbiota mainly by thriving under in fermentation systems. Most of the LAB metabolic traits are, actually, adaptations that contribute to its

competitive advantage in the sourdough environment (Gänzle and Ripari, 2016). Synthesis of bacteriocins probably contributes with a selective advantage in a microbial niche complex, such as sourdoughs (Vogel et al., 1993; Marques et al., 2017). Similarly to organic acids, an increase in mannitol production was observed from BS2 to BS4 (Menezes et al., 2019). Among LAB, only heterofermentative species are known to convert fructose into mannitol, including *L. curvatus* and *Ln. citreum* (Otgonbayar et al., 2011). The use of mannitol as external electron acceptors from fructose metabolism may lead to an efficient equilibration of the redox balance enhanced energy generation. Their production at the highest level during the first four to five days of propagation indicates their contribution to the strains' competitiveness when the ecosystem was still being established (Weckx et al., 2010a, 2010b).

The ecological concept of r- (copiotrophs) and K- (oligotrophs) selection can be applied to the kinetics of a microbial population (Koch, 2001; Pianka, 1970). Microorganisms classified as r-strategist show fast growth in environments with abundant nutrients, which are rapidly exploited, in its turn, k-strategists grow more slowly but using the limited resources more efficiently, are capable of surviving long periods of starvation (Fierer et al., 2007). Gram-negative bacteria and *Proteobacteria* are within the copiotrophic category, while Gram-positive bacteria are oligotrophic (Zhou et al., 2017). As for soil (Bastian et al., 2009; De Vries and Shade, 2013), the microbial communities in sourdoughs would be dominated by copiotrophic (r-strategists) in the early stages, while oligotrophs (K-strategists) increasing as the amount of substrate decreases in the final backslopping steps. K-strategists are presumably more efficient users of environmental resources that would be more competitive (Yang and Lou, 2011), and r-strategists would be expected to be dominant under low-stress conditions (Vasileiadis et al., 2015). This theory fits the dynamic observed on sourdough, with P*roteobacteria* and Gram-negative as *Enterobacteriaceae* being overcome by LAB through BS as the depletion of carbon sources, acidification, and redox potential make sourdough a stressful environment.

The temperature plays a key role for the sourdough ecosystem assembly and metabolite kinetics (Decock and Cappelle, 2005; Minervini et al., 2014; Vogelmann and Hertel, 2011b; Vrancken et al., 2011). Vrancken et al. (2011) demonstrated that microbial succession and the final composition of the microflora were different for temperature variations between 23 ºC and 30 ºC. Viiard et al. (2016)

observed that the ratio of bacterial species in rye sourdoughs propagated without temperature control was affected by the seasonal temperature fluctuations. Notably, the bacterial community between SD1 and SD2 differed over the final propagation steps. For SD1, LAB predominated while *Clostridium*, *Pseudomonas, and Enterobacteriaceae (Pantoea, Enterococcus, Enterobacter, K. cowanii* and *E. hermannii*) were gradually inhibited. From BS5 onward, only minor changes on microbiota were observed, indicating achievement of a stable microbial consortium, in agreement with the stabilization of the number of viable cells, TTA and pH (Menezes et al., 2019). In contrast, although the number of viable cells was stable from BS5 onward, the microbial community of SD2 remained unstable until BS9. Therefore, the stabilization of the counts, for temperature of 30 ºC ± 1ºC, cannot be the only parameter taken into account to predict that the microbial community is stable. Other authors (Minervini et al., 2012; Weckx et al., 2010b) found that the composition of sourdoughs microflora was always fluctuating, although bacterial and yeast counts and physical-chemical parameters were stable.

As for SD1, *Clostridium*, *Enterococcus*, and *Enterobacter* were inhibited at the final BS of SD2. However, *K. cowanii* and *E. hermannii* had increased their relative abundances and overcame *L. curvatus. L. farciminis* was reduced although remained predominant. *Pantoea* and *Pseudomonas* which had been reduced in BS4, increased in BS7 and BS9. These groups have the optimal growth temperature in the range of 30 to 37 ºC (Donnarumma et al., 2010; Rezzonico et al., 2009; Rogers et al., 2015) and are able to grow at a pH 4.0 (Rogers et al., 2015). As these groups were predominant in flour, and at each BS, they were again added to the sourdough. In SD2, they found favorable temperature for growth. Bessmeltseva et al. (2014) described a similar evolution for the microbial community for rye sourdough propagated at 20 and 30 ± 1 ºC. The rye flour was predominantly composed of *Proteobacteria*. After 24 h of fermentation, *Enterobacteriaceae* had dominated the dough, but LAB had already increased their relative abundance. After the third BS, enterobacteria were totally replaced by the LAB species for SD propagated at 20 ºC. On the other hand, enterobacteria were still present in low numbers within sourdoughs fermented at 30 ºC after the BS7.

The “Lacto” group had a significant negative correlation with the genera *Bacilllus*, *Clostridium*, *Escherichia*, and *Pseudomonas* for SD1. As the relative abundance of “Lacto” group increased, the other genera had their relative abundance significantly reduced, confirming the antagonistic relationship between these genera. This inhibitory effect

comprises and has already been observed in other microbial communities, as the human intestinal tract (Anand et al., 2018; Aoundia et al., 2016; Lei, Hsieh, Tsai, 2009; O'connor et al., 2015; Servin et al., 2004; Spinler, Ross, Savidge, 2016). It is an important tool that bases the biopreservation, applied in food systems to inhibit pathogenic and deteriorating microorganisms (Abdel-Rahman et al., 2019; Costa et al., 2018). The inhibitory effect was observed, however, only for SD1 propagated at 21 ± 1 ºC, indicating that temperature was an important factor shaping the microbial succession.

Although the SD had the same matrix until BS4, a variation in the temperature could change the composition of the final microbiota and, therefore, it would be able to modify the characteristics of the final product, as already reported in our previous study (Menezes et al., 2019). This consideration is pertinent for standardization of sensorial, nutritional and technological bread quality. The temperature of 30 °C can favor atypical bacterial groups, being inadequate for the propagation of sourdough in Brazil. This is the first study that investigated the relationship between temperature and the presence of groups of non-LAB bacteria, including potential pathogens, in wheat sourdoughs. Considering that the technology and functional fermentation performances are determined, among other factors, by the conditions of the process, as temperature, and the fermenting microbiota, the future research efforts should be dedicated to ensuring the consistent quality and safety of sourdoughs (Brandt, 2018; Gänzle and Zheng, 2018; Gobbetti et al., 2016). Evidently, to consider only one parameter at a time is not enough to fully explain the dynamics of the sourdough community. It is important not to neglect the fact that microbial growth is a result of multiple combinations of different parameters (Minervini et al., 2014), taking into account the complexity of the microbiota that composes a sourdough at different stages of propagation.

SD1 presented a lower diversity with LAB dominance. After nine BS, SD1 was metabolically and microbiologically stable. While SD2 still harboring atypical microorganisms. Supposedly, at a higher temperature, sourdough would take longer to achieve stability. Regardless of temperature, microbial diversity was markedly simplified after the BS5 for both SD. The highest bacterial diversity was detected for the first steps of propagation and gradually became lower as propagation progressed, finally reaching the lowest diversity in BS9. In general, microbial diversity tends to be simplified gradually through the BS (De Angelis et al., 2018). As the number of backslopping steps increases, the environmental conditions become more and more

selective, resulting in the dominance of a few species (Celano et al., 2016).

L. farciminis was dominant from BS4 until the end of the fermentation for both SD, regardless of temperature, which indicates a close adaptation to the nutritional restrictions and highly acidic conditions. This specie has already been isolated previously in sourdough, but is often not found frequently (De Vuyst et al., 2014; Galli et al., 2018; Gobbetti et al., 2016; Liu et al., 2016b). *L. farciminis* has a many carbohydrate subsystem features (Nam et al., 2011), including the Carbon Catabolite Repression (CCR), a major determining factor of growth rate and competitive success in natural ecosystems (Chen et al., 2018; Ganzle and Gobbetti, 2012). Furthermore, *L. farciminis* has multiple abilities to metabolize aminoacids, among them, the ADI-pathway, that contributes to production of ATP (Chiou et al. 2016), pH-homeostasis and acid tolerance (Fernández and Zúñiga, 2006). Galli et al. (2019) observed that, among five species of *Lactobacilli*, *L. farciminis* was the most competitive strain, increasing the cell numbers for the final BS, which reinforces the K-strategist concept.

Regarding microbial succession, the classic three-phase evolution (Ercolini et al., 2013; Van Der Meulen et al., 2007; Weckx et al., 2010b) was observed only for SD1. Atypical species for mature sourdoughs were detected only from BS1 to BS4. As the propagation steps evolved, more acidic conditions favored *Lactobacillus* over other LAB, that are species expected to be present for the initial steps of propagation, as they are more sensitive to acid stress (Van Der Meulen et al., 2007). On the other hand, For SD2, atypical bacteria were found to increase in the final BS. The presence of non-LAB bacteria in sourdoughs in previous studies might have been underestimated, since most research on sourdough microbial communities encompasses only LAB (Dertli et al., 2016; Lhomme et al, 2015; Liu et al., 2016; 2018; Scheirlinck et al., 2007; Van Der Meulen et al., 2007). Some recent studies have applied metagenetics to describe the populations, revealing the presence of persisting subpopulations, mainly *Enterobacteriaceae* (Bessmeltseva et al., 2014; Ercolini et al., 2013).

More than 50 species were detected from flour to BS9. When the microbial succession was studied by the culture-dependent approach, the number of isolated species was much lower. Discrepancies have been found between the results obtained by metagenomic analysis and isolate identification, whereby metagenomics tends to suggest a greater bacterial diversity (Michel et al., 2016). The culture-dependent approach

alone does not allow to detect all the bacteria present in complex matrices due to inherent limitations (Alfonzo et al., 2017). The number of isolates was probably not sufficient to completely describe the species and strain diversity; this also demonstrates a weakness of the culture-dependent approach. Microbial communities are highly diverse, community composition can change rapidly, and the vast majority of microbial taxa cannot be identified using standard culture-based methodologies. Metagenetics has the potential of giving a more detailed view on the micro-ecosystem composition, which will allow the expansion of classical models of ecological succession, as sourdough. Although it is not possible to distinguish intra-species variations, pyrosequencing enables the description of subdominant populations, which could hardly be studied through culture-dependent approaches. The subdominant population slightly affects the dough features, however, its effect should not be omitted (Van Der Meulen et al., 2007).

5. FINAL CONSIDERATIONS

The bacterial community of sourdoughs is showed to be affected by the temperature of propagation. *L. farciminis* is prevalent in both conditions tested; however, the temperature variation changed the subdominant populations. *L. farciminis* is not among the microorganisms most commonly found in European sourdoughs, however it was predominant in this study. The different processing conditions (temperature, flour origin) influenced the composition and dynamics of the microbial community, demonstrating the importance of studying sourdough in different parts of the world, as a source of microorganisms with new fermentative potentialities.

At 21 ± 1º C, the mature sourdough was composed exclusively by LAB, being able to inhibit the other bacterial groups as the propagation evolved. Otherwise, the temperature of 30 ± 1º C favored the persistence of atypical bacterial groups such as *Pseudomonas* and *Enterobacteriaceae* in the end of backslopping steps. The Pearson correlation demonstrated that there was an antagonistic relationship between *Lactobacillus* and *Lactococcus* and the genera *Bacillus, Clostridium, Escherichia, and Pseudomonas*. This effect was observed only at 21 ºC ± 1ºC. Therefore, the temperature of 21 ± 1º C can be considered more suitable for the propagation of sourdoughs in Brazil, since the role of non-LAB in sourdough metabolic activity is not yet well understood.

Most research has focused on identifying only LAB in sourdough. Hence, the presence of other groups and their putative contribution on fermentation has been neglected. Studies regarding microbial community dynamics of sourdoughs should advance the investigation into the presence of atypical microorganisms, including potentially pathogenic bacterial groups in mature sourdoughs and the consequent implications for baking, such as the production of metabolites and cross contamination in the bakery environment. In conclusion, the results reinforce i) temperature as a determining parameter for the microbial composition of sourdoughs and ii) temperature control as a fundamental criterion to ensure the quality of the sourdough bread.

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CHAPTER IV – USE OF SOURDOUGH FERMENTATION TO REDUCING FODMAPS IN BREADS

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USE OF SOURDOUGH FERMENTATION TO REDUCING FODMAPS IN BREAD

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Abstract. Fermentable, Oligosaccharides, Disaccharides, Monosaccharides And Polyols (FODMAPs) are a class of carbohydrates poorly digested that may trigger the symptoms of Irritable Bowel Syndrome (IBS) and Non-celiac Gluten Sensitivity (NCGS). The effects of sourdough fermentation on FODMAPs and organic acids were studied during the sourdough propagation and bread making. The concentrations of organic acids were higher for the first steps of propagation and became stable for final steps. All FODMAPs were significantly reduced during the propagation, except polyols. Sucrose, fructose and glucose were wholly degraded for the first step of fermentation. The other carbohydrates had their concentrations reduced after the fourth backslopping step. Sourdough bread presented the higher level of organic acids and polyols and lower content of fructans, sucrose, fructose and glucose than bread fermented by *Saccharomyces cerevisiae*. The fructans reduction was from 69 to 75%, indicating that sourdough fermentation can be applied for producing low-FODMAPs wheat bakery products.

Keywords: Short-chain Carbohydrates; Irritable Bowel Syndrome; Sourdough Bread; Fructans; Non-celiac Gluten Sensitivity; Lactobacilli.

1. INTRODUCTION

Sourdough results from the mixture of cereal flour and water, fermented mainly by lactic acid bacteria (LAB) and yeasts, which can grow spontaneously, from the flour and other ingredients, or can be added as a starter culture (Gobbetti et al., 2014; Minervini et al., 2014). Several studies have confirmed the ability of sourdough fermentation to promote beneficial modifications on sensory (Pétel, Onno, & Prost, 2017), technological and nutritional properties (Gobbetti et al., 2014; Rinaldi et al., 2017; Torrieri et al., 2014) of leavened baked goods. These effects are, however, dependent on the microbial composition of sourdough (Ganzle, 2014), which is modulated by some parameters as time and temperature of fermentation and type and origin of flour (Minervini et al., 2014).

The more expressive metabolic activities of microbial communities during fermentation are acidification, leavening, and flavor formation (Van Kerrebroeck et al., 2016), all related to metabolism of carbohydrates. Conversions involving hexoses and pentoses are well explored in the literature (Dertli et al., 2016), however, fermentation of other saccharides remains little known. Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols (FODMAPs), are an heterogeneous class of compounds (most of which are short-chain carbohydrates) that includes lactose, fructose in excess of glucose, fructans and fructooligosaccharides (FOS, nystose, kestose), raffinose family of oligosaccharides (GOS, such as raffinose and stachyose), and sugar polyols (sorbitol, mannitol) (Muir et al., 2009). This group can be poorly absorbed by the small intestine and may have a wide range of effects on gastrointestinal processes, including a key role in triggering the Irritable Bowel Syndrome (IBS) symptoms (Menezes et al., 2018).

IBS is one of the most common types of functional bowel disorders; the prevalence is estimated between 7 and 21% worldwide (Brandt et al., 2009; Lovell & Ford, 2012). Typical IBS symptoms are abdominal discomfort and pain, changes in bowel habits, diarrhea, constipation, bloating, distension, and flatulence (De Giorgio, Volta, & Gibson, 2016; Tuck et al., 2014). The low-FODMAPs diet has been related as one of the most effective dietary therapies for IBS (Zannini & Arendt, 2018). Recently, FODMAPs have been implicated with the onset of the symptoms of Non-celiac Gluten Sensitivity (NCGS) (Dieterich et al., 2018; Skodje et al., 2018), a condition characterized by gastrointestinal and extra-intestinal symptoms in the absence of celiac disease or wheat allergy. The estimated prevalence of NCGC ranges

from 0.6 to 6% of the general population, and usually occurs in 25-30% of IBS patients (Henggeler, Veríssimo & Ramos, 2017). In fact, FODMAP restriction has shown positive results also in NCGS treatment (Biesiekierski et al., 2013; Halmos et al. 2014).

Due to high daily consumption in many countries worldwide, bread represents a significant proportion of FODMAPs intake. Fructans are the main FODMAPs in wheat-based products (Menezes et al., 2018, Biesiekierski et al., 2011; Shewry & Hey, 2015; Ziegler et al., 2016). In this study, for the first time, the effects of sourdough fermentation on FODMAPs and acidification kinetics were investigated during sourdough propagation and in bread making. The targeted results should contribute to the investigation of sourdough ability to reduce the concentration of FODMAPs in bread, as an alternative for producing low-FODMAPs wheat bakery products, suitable for IBS- and NCGS-sufferers.

2. MATERIALS AND METHODS

2.1 SAMPLES, CHEMICALS AND STANDARDS

Commercial organic wheat flour was purchased from Paullinia company (Marechal Cândido Rondon, Paraná, Brazil). Mineral water, refined salt and commercial fresh baker's yeast (lyophilized *Saccharomyces cerevisiae*) were purchased in a local market. All standards were in analytical grade (minimum 98% purity). Sorbitol (SBL), mannitol (MAN), glucose (GLU), fructose (FRU), sucrose (SUC), raffinose (RAF), stachyose (STA), 1-kestose (KES), and 1-nystose (NYS), potassium sorbate (SOR), citric (CTA), lactic (LCA), propionic (PPA) and benzoic (BEN) acids were supplied by Sigma–Aldrich (Taufkirchen, Germany). Acetic acid (ACA) was supplied from Tedia Co. (Fairfield, OH, USA).

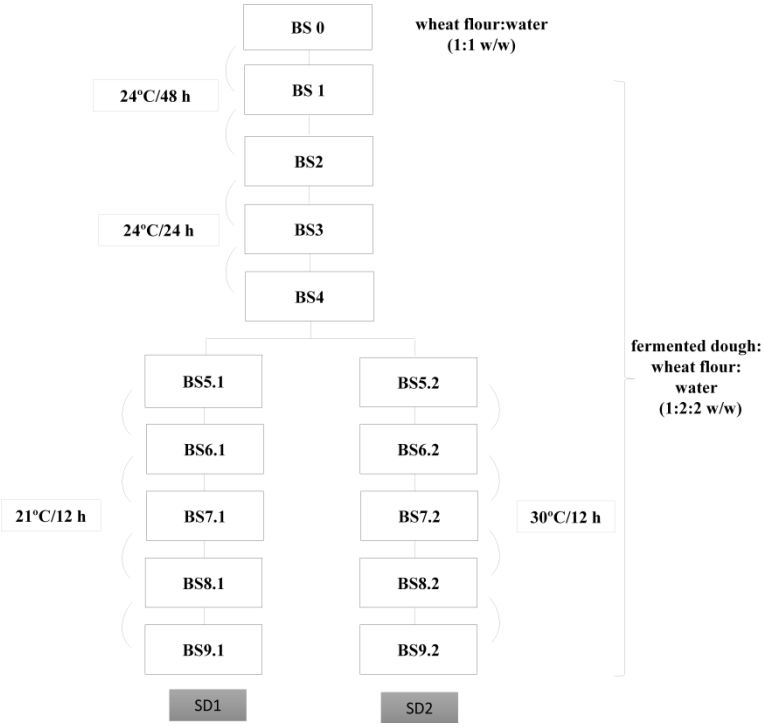
All solvents were in chromatographic grade. Ultra-pure water was obtained from a Mega Purity water purification system (Billerica, MA, USA). Merck KGaA (Darmstadt, Germany) supplied Acetonitrile (ACN) and methanol (MET). Formic acid (FMA) was obtained from J.T. Baker Chemical Co. (Taufkirchen, Germany). Phosphoric acid (PHA) was supplied by Sigma–Aldrich (Taufkirchen, Germany). Stock solutions (1,000 mg L^{-1}) of SBL, MAN, GLU, FRU, SUC, RAF, STA, KES, SOR, CTA, LCA, PPA, and BEN were prepared by dissolving the standards in methanol with low proportions of water. The stock solution for NIS (1,000 mg L^{-1}) was prepared by dissolving the standard in

acetonitrile with 0.1% formic acid. Working solutions (100 mg L^{-1}) were prepared by diluting all stock solutions with methanol. All solutions were cold stored (−20 ± 10 °C).

2.2 SOURDOUGH PROPAGATION

Sourdoughs (SD) were made and propagated through traditional protocol - sourdough type I (Figure 1).

Figure 1. Set-up of fermentations.



Two distinct and common temperatures in tropical climates were selected for sourdough preparation. Doughs were prepared by mixing wheat flour and water [(1:1 (w/w)] with a resulting dough yield [(dough mass/flour mass) × 100] of 200 and incubated at 24 °C for 48 h. After this first fermentation (backslopping one - BS1), eight backslopping steps (BS2 to BS9) were further carried out. Initially, a

portion of fermented dough (FD) was harvested and used as an inoculum for a subsequent backslopping, mixed with wheat flour and water [FD: water:wheat:flour (1:2:2 w/w)] and incubated at 24 °C for 48 h again (BS2). For BS3 and BS4, wheat flour and water were mixed with the FD and stored at 24 °C for 24 h. After that, the FD was fractionated in two portions; the first one was incubated at 21 ºC ± 1ºC (Sourdough one - SD1) and the second one at 30 ºC ± 1ºC (Sourdough two - SD2). Subsequently, five BSs were further carried out mixing FD:water:wheat flour (1:2:2 w/w) at 12 h intervals.

2.3 LAB AND YEASTS ENUMERATION

Ten grams of the flour and BS samples were homogenized by adding 90 ml 0.1% (w/v) of sterile peptone solution using a vortex. A 10-fold dilution series were made and plated in mMRS (Man, Rogosa, Sharpe agar medium, modified by addition of maltose 1%) at 37 ºC for 48 h in anaerobic conditions (Lhomme et al. (2015) and YEPD (Yeast Extract-Peptone-Dextrose agar medium) (Li; Li; Bian, 2016) at 25 ºC for 48 h. The results were expressed as log CFU g^{-1}.

2.4 BREAD MAKING

Four trials were prepared for bread making. For trials I and II, sourdoughs (SD1 and SD2) were used as starters. For experiment III (SC1) and IV (SC2), sourdough was replaced by fresh baker's yeast. Flour (58.1 % w/w), salt (1.2% w/w), water (34.8% w/w), and the respective leavening agent (8.7% w/w) were mixed for 10 min. SD1, SD2, and SC1 were incubated at room temperature (~25 ºC), for 10 h. Subsequently, the doughs were portioned, modeled, and incubated for 2 h at room temperature. SC2 was prepared according to the baker's yeast manufacturer's recommendation – the ingredients were mixed and the dough was incubated until the volume was doubled (~ 3 h). The breads were baked at 180 ºC in a combined electric oven (Turbo ftt-120, Tedesco, Caxias do Sul, Brazil) for 30 min in combined heat, and 20 min in dry heat.

2.5 TOTAL TITRATABLE ACIDITY (TTA) AND PH

The pH value of doughs was determined by a pH meter (Del Lab, São Paulo, Brazil). TTA was determined after homogenization of 10 g of dough with 90 mL of distilled water and expressed as the amount

(mL) of NaOH 0.1 mol L^{-1} required to neutralize the solution, using phenolphthalein as indicator (official AACC method 02-31.01).

2.6 ORGANIC ACIDS AND *FODMAPS*

2.6.1 Method of extraction

For the extraction of FODMAPs, CTA, LCA, BEN, and SOR, the sample was weighed (2.0 ± 0.1 g) into a 50 mL polypropylene tube. Subsequently, 10 mL of extraction solution (formic acid 0.1% in water:methanol 1:9, v/v) were added to each tube. For ACA and PPA, 5.0 ± 0.1 g of sample were weighed into polypropylene tubes, and the extraction solution was water:methanol (1:1, v/v). The suspension was mildly shaken on an orbital shaker for 10 min and then centrifuged at 3,488 G-force for 20 min at 4°C. The supernatants were transferred to 15 mL polypropylene tubes which have been kept at −20 °C for an hour. Centrifugation was performed again in the same conditions described above. Finally, an aliquot of 10 µL of the extract was diluted in 990 µL initial mobile phase and transferred to a 1.5 mL polypropylene microtube, centrifuged at 17,530 G-force for 10 min. The extract was transferred to an autosampler vial and then injected into the chromatograph system.

2.6.2 Instrumental

ACA and PPA - Liquid chromatography was performed on an Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) with a quaternary gradient pump, column heater and photodiode array detector (PDA) (Waters Corporation 2998). Empower data software from the equipment constructor was used for data processing.

FODMAPs, SOR, BEN, CTA and LCA - A 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Sciex, Framingham, MA, USA), equipped with an electrospray ionization (ESI) source, working in the positive and negative modes and multiple-reaction monitoring (MRM) mode. The spectrometer was coupled to a 1290 Infinity high-performance liquid chromatography system (HPLC) from Agilent Technologies Inc. (Santa Clara, CA, USA). The Analyst and the MultiQuant softwares (Sciex, Framingham, MA, USA) performed all system control, data acquisition, and data analysis.

2.6.3 Liquid chromatography and mass spectrometry

ACA and PPA – The mobile phase was composed by an aqueous solution with 0.1% phosphoric acid. The detection wavelengths were 210 and 230 nm. Separations were conducted with a ZORBAX C18 column (250 × 4.6 mm i.d.; 5 µm particle size) (Agilent Technologies, Wilmington, DE, USA), maintained at 30°C. The flow rate was 1,000 µL/min, and the injection volume was 20 µL.

SOR, BEN, and CTA – Chromatographic separation was performed using stationary phase (Zorbax 300 SB-CN, 150 mm x 4.6 mm i.d., 5 µm particle size, 300 Å) (Agilent Technologies, Inc., Santa Clara, CA, USA) according to Molognoni et al. (2018). The mobile phase consisted of the aqueous solution with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1% formic acid (mobile phase B). The linear gradient elution was performed as follows: 0-1 min 90% A; 2-3 min 80% A; 4 min 70% A; 5-6 min 50% A; 7-8 min 10 % A; 9 min 50% A; 10 min 90% A and held for 4 min for system auto-equilibration. The column was maintained at 40°C. The flow rate was 0.5 mL/min, and the injection volume was 10 µL.

LCA and FODMAPs – The mobile phase consisted of an aqueous solution with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1% formic acid (mobile phase B). For LCA, the linear gradient elution was performed as follows: 0-2 min 95% A; 2-3 min 80% A; 3-5 min 10% A; 5-7 min 90% A; 7-10 min 95% A. Chromatographic separation of LCA was performed using a ZORBAX C18 column (150 mm × 2.1 mm i. d.; 3.5-µm particle diameter) from Agilent Technologies, according to Molognoni et al. (2016) with some modifications. Chromatographic separation of FODMAPs was performed using a Nucleosil-NH2 column (250 mm × 4.6 mm i.d., 5 µm particle size) (Phenomenex Inc., Torrance, CA, USA), according to Liu, & Rochfort (2015) with modifications. The columns were maintained at 30°C. The flow rate was 500 µL/min, and the injection volume was 10 µL. For FODMAPs, the linear gradient elution was performed as follows: 0-1 min 95% A; 1-3 min 10% A; 3-5 min 15% A; 5-7 min 20% A; 7-9 min 25% A; 9-11 min 30% A; 11-13 min 35% A; 13-15 min 40% A; 15-20 min 50% A, and held for 3 min to equilibrate the column. The column was maintained at 30 °C. The flow rate was 1,000 µL/min, and the injection volume was 15 µL.

For all analytes, a matrix-matched analytical curve was prepared with six concentration levels (including zero), using a linear function of concentration (x) versus peak area (y). For this, the blank sample (wheat

flour extract) was fortified with the desired amounts of standards. The measuring range of calibration curve was 2.50-20.0 mg/L to ACA and PPA, 0.25-2.00 mg/L to LCA, 2.5-10 mg/L to SOR, 2.50-10.0 mg/L to BEN, and 0.10-2.50 mg/kg to FODMAPs.

The optimization of the mass spectrometer in MRM was obtained by the infusion of the compounds separately in the ESI-MS/MS (mass spectrometer), in a continuous flow of 10 µL/min at concentrations of 10-150 µg/L. The optimization of the ESI source in both positive and negative modes was carried out by flow injection analysis according to the following parameters, for FODMAPS and other analytes, respectively: source temperature (TEM) 650 ºC, 550 ºC; collision gas (CAD) high for both; voltage capillary (IS) 4500 for both; nebulizer gas (GS1) 55 psi, 50 psi; drying gas (GS2) 55 psi, 55 psi; curtain gas (CUR), 30 psi, 25 psi. The optimization of each parameter in the MS was performed to obtain maximum sensitivity. The optimization of the ESI in positive and negative modes is shown in table supplementary 1 (TS1).

2.7 TOTAL FRUCTANS

Megazyme Fructan HK enzymatic assay kit (Megazyme, Bray, Ireland) was used for the determination of total fructan contents in all samples. The enzymatic assay was based on established standard methods (AOAC method 999.03 and AACC method 32.32.01).

2.8 STATICAL ANALYSIS

Data were subjected to one-way ANOVA; pair-comparison of treatment means was obtained by Tukey's procedure at $p < 0.05$, using the statistical software *Statistica* 11.0 (StatSoft Inc., Tulsa, USA).

3. RESULTS

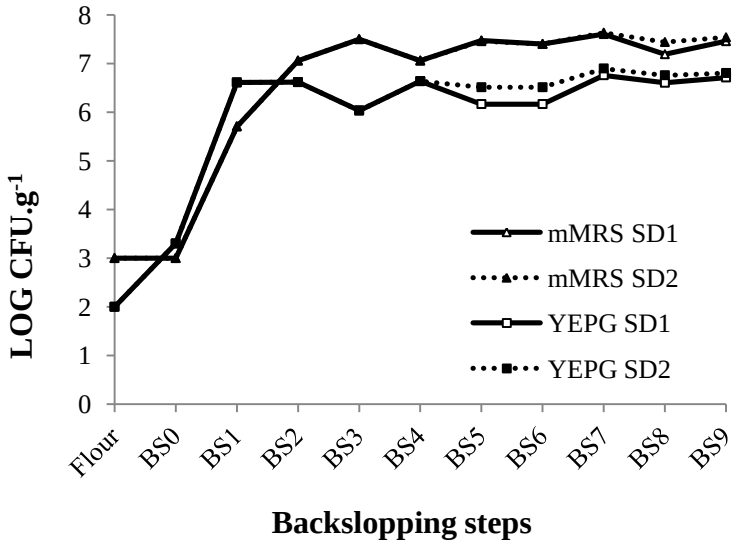
3.1 SOURDOUGH PROPAGATION

3.1.1 LAB and yeasts enumeration

The presumptive LAB counts in mMRS for flour and before fermentation (BS0) were 3.0 CFU g^{-1} (Figure 2). After 48 h of fermentation (BS1), the cell density of LAB in significantly increased ($p < 0.05$) to 5.7 log CFU g^{-1}. From BS2 (four days of fermentation) onward, the counts reached 7.1 log CFU g^{-1} SD and stayed almost

constant during subsequent propagations. For BS9, the number of LAB was 7.5 log CFU g^{-1} for both SD. Flour contained a low cell density of yeasts (2.0 log CFU g^{-1}), which significantly ($p < 0.05$) increased after the first 48 h of fermentation (6.6 log CFU g^{-1} for BS1). For BS7, the counts reached the maximum value for both SD (6.8 and 6.9 log CFU g^{-1}, for SD1 and SD2, respectively). For the final step, the cell density of yeast was 6.7 for SD1 and 6.8 log CFU $g^{-1}$for SD2.

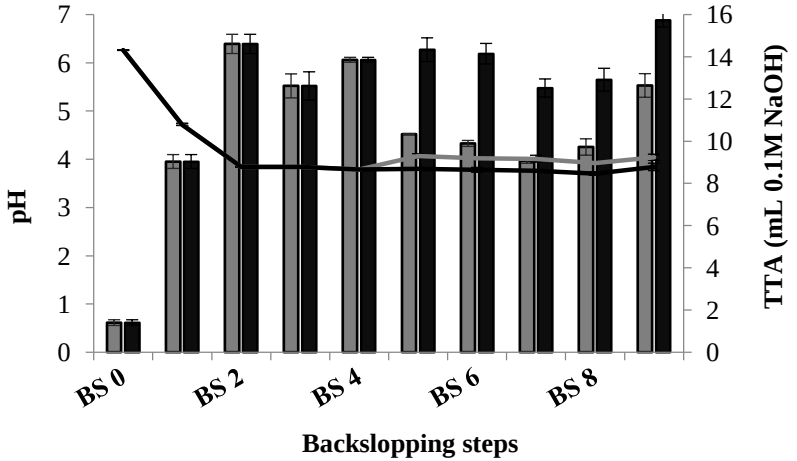
Figure 2. Cell density of LAB (mMRS) and yeasts (YEPD) (log CFU g^{-1}) for each backslopping step (BS) during sourdough (SD) propagation. Triangles: mMRS; Squares: YEPD; Solid line: SD1 (21 °C); Dashed line: SD2 (30 °C).



3.1.2 TTA, pH and organic acids

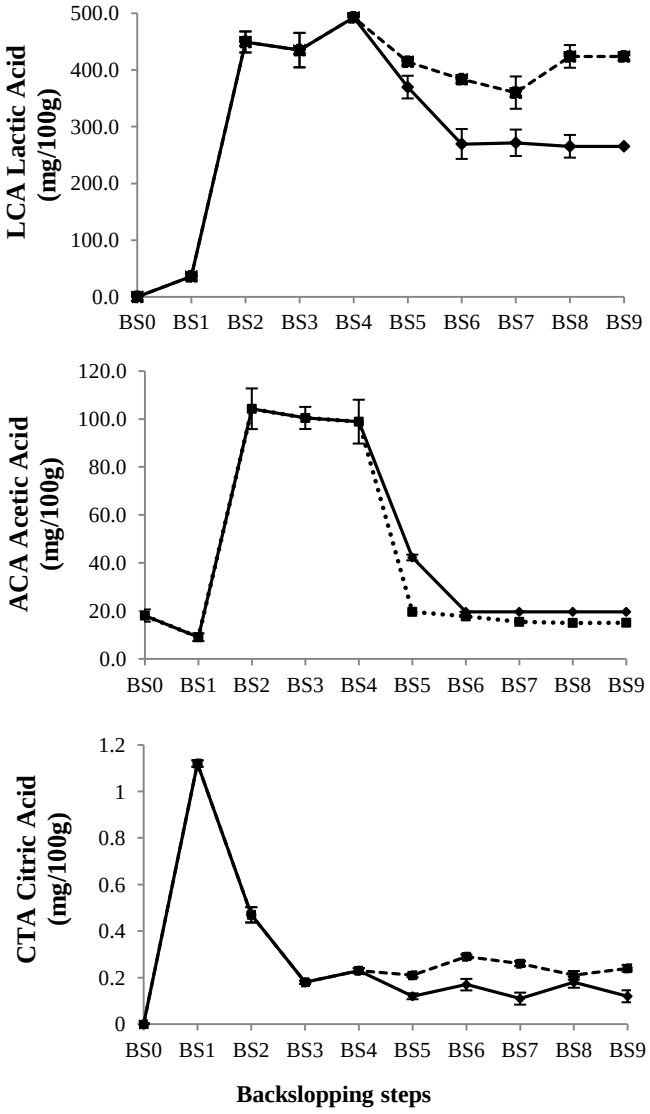
The initial pH was 6.26 ± 0.05 for the unfermented dough (BS0) (Figure 3). After a sharp drop at BS2 (3.84), the values were kept constant from the BS5 onward to SD1 and BS3 to SD2. At the end of the set-up of fermentations, the pH was 4.04 for SD1 and 3.84 for SD2. TTA increased from 1.40 mL for the unfermented dough (BS0) to 12.63 mL for SD1 and 15.73 mL for SD2 at BS9.

Figure 3. Course of pH (Grey line: SD1 21°C; Black line: SD2 30°C) and TTA (Grey bars: SD1 21°C; Black bars: SD2 30°C) during the backslopping steps (BS) of sourdough (SD) propagation.



ACA concentration at BS0 was 9.00 mg/100 g, increased to 104.42 mg/100 g at BS2 and remained high up to BS4 (98.87 mg/100 g). Afterwards, the concentrations decreased. From BS5 onward, the amount of ACA was stable for both SD. The final concentration was highest for SD1 (19.59 mg/100 g and 15.07 mg/100 g for SD2) (Figure 4). The concentration of LCA was 0.005 mg/100 g for BS0. For SD1, concentrations decreased from BS4 to BS6 and then remained stable. For SD2, a less pronounced drop was observed until the BS7 then the concentration increased again. A slight increase was observed for BS1 (36.19 mg/100 g), then the highest concentrations were observed between BS2 (448.93 mg/100 g) and BS4 (492.37 mg/100 g). At the end of propagation, SD1 contained 265.2 mg/100 g of LCA while SD2 contained 423.5 mg/100 g. Regardin CTA, after an initial increase (from 0.02 mg/100 g for BS0 to 1.12 mg/100 g for BS1), the amounts decreased for BS3 (0.47 mg/100 g) and BS4 (0.18 mg/100 g), and subsequently, despite slight fluctuations, the concentration kept constant until BS9 (final concentration 0.12 for SD1 mg/100 g; 0.24 mg/100 g for SD2), PPA, SOR, and BEN were not detectable for any sample (<0.25 mg/100 g to PPA an SOR, and <0.31 mg/100 g to BEN).

Figure 4. Organic acids through backslopping steps (BS). Solid line: SD1 21°C; Dashed line SD2 30°C.

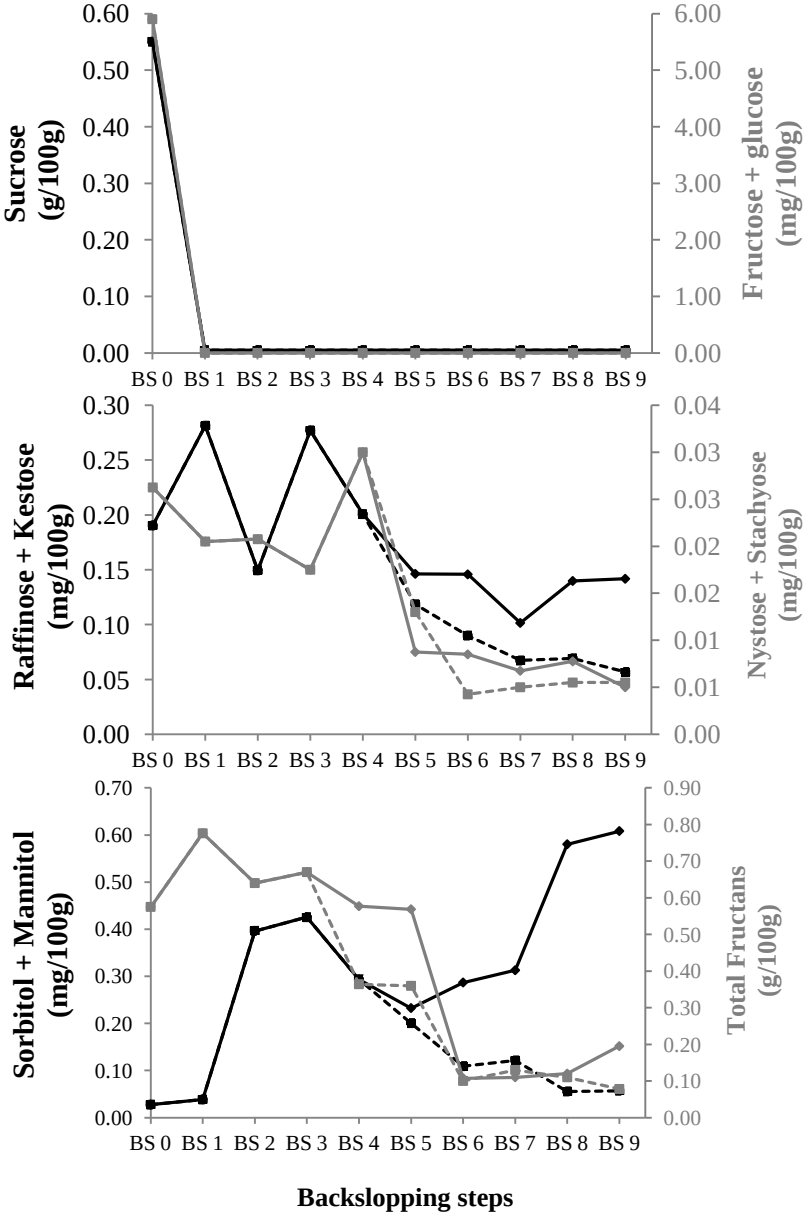


3.1.3 FODMAPs

Both sourdoughs presented the ability to reduce FODMAPs during the backslopping steps (Figure 5). The content of SUC and FRU + GLU for BS0 was 0.55 g/100 g and 5.90 mg/100 g, respectively. These carbohydrates are consumed since the first stage of sourdough fermentation. After BS1, these concentrations dropped below the limits of quantification (< 0.1 mg/kg). The other subgroups of FODMAPs have their concentrations reduced later, after BS3 (RAF + KES; SOR + MAN for SD1) and BS4 (NYS + STA; total fructans). RAF + KES levels ranged from 0.19 mg/100 g for BS0 to 0.14 for SD1 and 0.01 for SD2, for BS9, reductions of 24% for SD1 and 95% for SD2. The concentration of NYS + STA reduced 81% for SD1 and 79% for SD2. The value found for BS0 was 0.13 mg/100 g while for BS9 was around 0.01 mg/100 g for both sourdoughs. Total fructans content reduced 65% for SD1 and 82% for SD2, dropping from 0.58 g/100 g for BS0 to 0.20 for SD1 and 0.08 for SD2, at the end of propagation.

SOR + MAN concentrations increased from the BS0 to BS3 (from 0.03 mg/100 g to 0.43 mg/100 g), and then, the sourdoughs presented different behaviors. A gradual decrease was observed from BS3 to the end of propagation for SD2 (final concentration 0.06 mg/100 g), while SD1 presented a reduction of polyols from BS3 to BS5, and a significant increase from BS6 forward (final concentration 0.61 mg/100 g for BS9).

Figure 5. FODMAPs concentration throughout backslopping steps (BS). Solid line: SD1 21°C; Dashed line SD2 30°C. black lines: left axis; Gray lines: right axis.



3.2 BREAD MAKING

3.2.1 LAB and yeasts enumeration

Before fermentation, the number of yeasts was 5.14 log CFU g^{-1} for SD1 and 6.03 log CFU g^{-1} for SD2. At the end of fermentation, the cell density was 6.46 log CFU g^{-1} for SD1 and 6.65 log CFU g^{-1} for SD2 (an increase of 0.97 log CFU g^{-1} and 0.25 log CFU g^{-1}, respectively). Regarding LAB, the unfermented doughs harbored 6.46 log CFU.g^{-1} and 6.65 log CFU.g^{-1}, for SD1 and SD2, respectively. After 12 h fermenting, an increase in cell density of LAB was also detected (7.47 log CFU $g^{-1}$and for SD1 and 7.44 log CFU g^{-1} for SD2, corresponding to an increase of 1.02 log CFU g^{-1} and 0.79 log CFU g^{-1}, respectively).

3.1.2 TTA, pH and organic acids

TTA and pH changed sharply and similarly for the doughs fermented with sourdough SD1 and SD2 (Table 1). The values of pH and TTA of doughs prepared with *S. cerevisiae* (SC1 and SC2) discreetly changed during fermentation. Compared to *S. cerevisiae*, sourdough fermentation caused a marked increase of TTA, attaining a concentration 3-fold higher. Sourdough starter showed higher acidifying capacity than *S. cerevisiae* during the fermentation. Sourdough breads had significantly ($p < 0.05$) higher TTA and lower pH than breads fermented with *S. cerevisiae*. The pH of both SD breads did not differ statistically from each other, however, TTA of SD breads was significantly higher for SD1. There was no significant difference between SC1 and SC2 for pH and TTA.

Accumulation of organic acids during bread making was markedly higher for the doughs fermented with sourdough (Figure 6). The concentration of acetic acid increased about 3.5-fold for SD1 and 2-fold for SD2. The increase of LCA was 8% and 5% for SD1 and SD2, respectively. For SC1, this increase was 3%. Despite this increase, the concentration of LCA for the SD doughs was markedly higher. Accumulation of CTA during fermentation was very low for SC doughs, while for SD doughs the increase was around 5 to 6-fold. After baking, SD breads had concentrations of LCA (9.67 mg/100g for SD1 and 8.58 mg/100g for SD2) at least10-fold higher than SC bread (0.12 mg/100g for SC1 and 0.86 mg/100g for SC2). SD1 presented the higher amounts of ACA (24.92 mg/100g), followed by SC2 (15.67 mg/100g). For the others, the amounts were similar (11.09 mg/100g for SD2, 11.81

mg/100g for SC1). No CTA was found for SC1 and SC2, while SD1 had 0.72 mg/100g and SD2 had 0.63 mg/100g.

Table 1. pH and total titratable acidity (TTA) values during bread making.

	pH		
	UD	FD	Breads
SD1	6.07 ± 0.03^{b}	5.44 ± 0.01^{b}	4.42 ± 0.11^{b}
SD2	5.98 ± 0.05^{c}	5.49 ± 0.05^{b}	4.48 ± 0.01^{b}
SC1	6.35 ± 0.07^{a}	6.31 ± 0.02^{a}	6.23 ± 0.30^{a}
SC2	6.32 ± 0.04^{a}	6.27 ± 0.03^{a}	6.22 ± 0.28^{a}
	TTA*		
	UD	FD	Breads
SD1	2.49 ± 0.26^{a}	5.14 ± 0.24^{a}	6.96 ± 0.65^{a}
SD2	1.92 ± 0.26^{b}	5.04 ± 0.11^{a}	5.68 ± 0.67^{b}
SC1	1.06 ± 0.13^{c}	1.82 ± 0.13^{b}	3.15 ± 0.13^{c}
SC2	1.05 ± 0.13^{c}	1.24 ± 0.14^{c}	3.14 ± 0.14^{c}

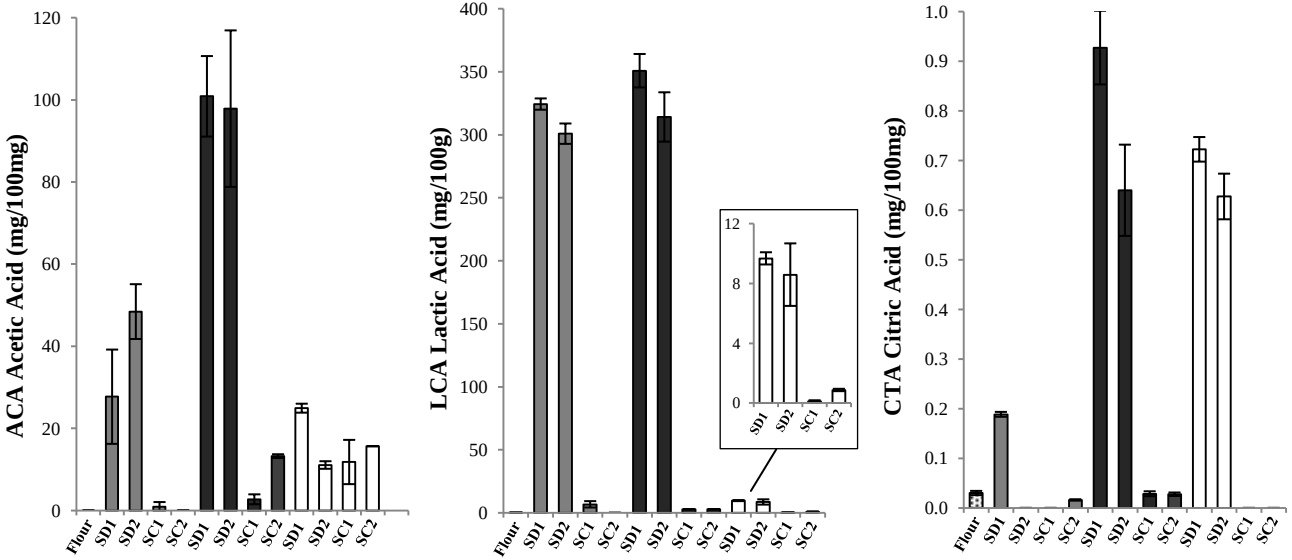
[a-b] Values in the same column with different superscript letters differ significantly *($p < 0.05$)*

[*] mL of 0.1 M NaOH/10 g.

[**] Doughs and breads fermented with: Sourdough 1 (21 ºC± 1ºC) (SD1); Sourdough 2 (30 ºC ± 1ºC) (SD2). *S. cerevisiae* (12 h) (SC1); *S. cerevisiae* (3 h) (SC2).

(UD) Unfermented doughs, the dough before fermentation. (FD) Fermented doughs, the doughs after fermentation, 3 h for SC2 and 12 h for SD1, SD2 and SC1.

Figure 6. Organic acids for doughs and breads fermented with Sourdough 1 21 ºC ± 1ºC (SD1), Sourdough 2 30 ºC ± 1ºC (SD2), *S. cerevisiae* (12 h) (SC1) and *S. cerevisiae* (3 h) (SC2). Dotted bars: flour. Grey bars: unfermented doughs; Black bars: fermented doughs; White bars: breads.



3.2.3 FODMAPs

During bread making, concentrations of SUC, FRU + GLU, and fructans were significantly ($p < 0.05$) reduced after fermentation for sourdough breads (Table 2). SD1 and SD2 presented higher capacity to degrade SUC, FRU + GLU and fructans compared to *S. cerevisiae*. On the other hand, amounts of SOR + MAN increased significantly for SD1 and SD2. There was no increasing of polyols for the doughs fermented by *S. cerevisiae*, which did not differ from each other. RAF + KES concentration also increased for SD doughs. Reduction in fructans was 69% for SD1 and 75% for SD2. There was no significant reduction of fructans during the fermentation of SC1 and SC2. Sourdough breads presented lower concentration of SUC, FRU + GLU, and fructans than the SC breads (Table 3). The highest concentrations of polyols were found for SD1, while SD2 showed lower FRU + GLU and fructans. The amount of fructans for SD breads was 40 to 62% lower than *S. cerevisiae* bread fermented by traditional protocols (SC2). There was no statistic difference for the total content of fructans between both SC breads. No statistical differences were found between treatments for RAF + KES and NYS + STA.

Table 2. Content of FODMAPs for doughs, prepared using sourdough and baker's yeast.

	Sucrose (g/100g)		Fructose + Glucose (mg/100g)		Sorbitol + Mannitol (mg/100g)		Raffinose + Kestose (mg/100g)		Nystose + Stachyose (mg/kg)		Fructans (g/100g)	
	UD	FD	UD	FD	UD	FD	UD	FD	UD	FD	UD	FD
SD!	$0.31\pm0.00^{A,a}$	$0.00\pm0.00^{B,b}$	$106.27\pm5.58^{B,a}$	$19.2\pm1.40^{A,b}$	$0.33\pm0.08^{A,a}$	$0.90\pm0.14^{A,b}$	$0.07\pm0.02^{A,b}$	$0.15\pm0.01^{A,a}$	$0.07\pm0.06^{A,a}$	$0.01\pm0.00^{A,b}$	$1.12\pm0.19^{A,a}$	$0.35\pm0.14^{B,b}$
SD2	$0.34\pm0.02^{A,a}$	$0.00\pm0.00^{B,b}$	$106.27\pm5.58^{B,a}$	$32.76\pm0.81^{A,b}$	$0.04\pm0.01^{B,b}$	$0.44\pm0.06^{B,a}$	$0.05\pm0.01^{A,b}$	$0.11\pm0.02^{A,a}$	$0.01\pm0.00^{A,a}$	$0.01\pm0.01^{A,a}$	$1.10\pm0.08^{A,a}$	$0.27\pm0.09^{B,b}$
SC1	$0.28\pm0.00^{A,a}$	$0.04\pm0.00^{A,b}$	$191.69\pm1.62^{A,a}$	$10.00\pm .26^{A,b}$	$0.03\pm0.01^{B,a}$	$0.05\pm0.01^{C,a}$	$0.03\pm0.00^{A,b}$	$0.07\pm0.00^{A,a}$	$0.01\pm0.00^{A,a}$	$0.01\pm0.00^{A,a}$	$1.18\pm0.11^{A,a}$	$1.00\pm0.05^{A,a}$
SC2	$0.39\pm0.01^{A,a}$	$0.08\pm0.00^{A,b}$	$191.69\pm1.62^{A,a}$	$20.50\pm0.53^{A,b}$	$0.04\pm0.00^{B,a}$	$0.04\pm0.00^{C,a}$	$0.04\pm0.01^{A,b}$	$0.04\pm0.02^{A,a}$	$0.01\pm0.00^{A,a}$	$0.01\pm0.01^{A,a}$	$1.19\pm0.12^{A,a}$	$1.15\pm0.15^{A,a}$

$^{A-B}$ Values in the same column with different superscript letters differ significantly *($p < 0.05$)*

$^{a-b}$ Values in the same row with different superscript letters differ significantly *($p < 0.05$)*

Doughs fermented with: Sourdough 1 (21 ºC± 1ºC) (SD1); Sourdough 2 (30 ºC ± 1ºC) (SD2). *S. cerevisiae* (12 h) (SC1); *S. cerevisiae* (3 h) (SC2).

(UD) Unfermented doughs, the dough before fermentation. (FD) Fermented doughs, the doughs after fermentation, 3 h for SC2 and 12 h for SD1, SD2 and SC1.

Table 3. Content of FODMAPs for wheat breads, prepared with fermentation using sourdough and baker's yeast.

	Sucrose (g/100g)	Fructose + Glucose (mg/100g)	Sorbitol + Mannitol (mg/100g)	Raffinose + Kestose (mg/100g)	Nystose + Stachyose (mg/kg)	Total Fructans (g/100g)
SD[1*]	0.37 ± 0.02[a,b]	60.65 ± 0.79[b]	1.00 ± 0.29[a]	0.10 ± 0.00[a]	0.01 ± 0.00[a]	0.29 ± 0.05[b]
SD2[*]	0.34 ± 0.02[b]	40.40 ± 0.05[c]	0.52 ± 0.14[a,b]	0.09 ± 0.01[a]	0.03 ± 0.01[a]	0.19 ± 0.07[c]
SC1[*]	0.42 ± 0.02[a]	91.72 ± 0.69[a]	0.05 ± 0.00[b]	0.07 ± 0.02[a]	0.01 ± 0.00[a]	0.40 ± 0.08[b]
SC2[*]	0.42 ± 0.02[a]	90.39 ± 1.21[a]	0.05 ± 0.00[b]	0.05 ± 0.01[a]	0.03 ± 0.02[a]	0.49 ± 0.10[a,b]
Flour	0.55 ± 0.03	11.80 ± 0.76	0.06 ± 0.00	0.38 ± 0.05	0.26 ± 0.02	1.20 ± 0.26

[a-b] Values in the same column with different superscript letters differ significantly *($p < 0.05$)*

[*] Breads fermented with: Sourdough 1 (21 ºC ± 1ºC) (SD1); Sourdough 2 (30 ºC ± 1ºC) (SD2). *S. cerevisiae* (12 h) (SC1); *S. cerevisiae* (3 h) (SC2).

4. DISCUSSION

Several parameters drive the dynamics of the sourdough microbial community, among them, the temperature of fermentation (Minervini et al., 2014). Sourdough fermentation was followed for the first time in Brazil, a country with predominantly tropical climate. Vrancken et al. (2011) demonstrated that microbial dynamics and consequently, the final composition of the microflora were different for temperature variations between 23 ºC and 30 ºC. The first microbial community of sourdough corresponds to the autochthonous microorganisms of flour and other ingredients, consisting of LAB, aerobic bacteria, *Enterobacteriaceae*, yeasts and moulds, usually below 5 log CFU g^{-1}. Over the consecutive fermentation steps, taking into account physic-chemical characteristics like water activity (aw), pH and redox potential, and the ability to use of the available substrates, sourdough allows LAB and yeast to outgrow other microbial populations (Ercolini et al., 2013; Minervini et al., 2014; Scheirlinck et al., 2007; Van der Meulen et al., 2007). A stable number of yeasts and LAB was observed from the fifth day of propagation onward. Other studies (Van der Meulen et al., 2007; Ercolini et al., 2013; Vrancken et al., 2011) showed that sourdoughs became mature within one week, with slight variations. Commonly, LAB are predominant in wheat flour mature sourdoughs (Minervini et al. 2015; Vogelman et al., 2009; Zhang et al. 2011) and the ratio between LAB and yeasts can range from 10,000:1 to 10:1 (Ercolini et al., 2013; Gobbetti et al., 2016, Lhomme et al., 2015).

A sharp drop of the pH and an increase of the TTA were observed for the initial steps of the propagation (BS1-BS4), until relatively stable values were reached after several steps. The highest concentrations of organic acids (BS1-BS4) coincided with the exponential growth phase of the sourdough community. Synthesis of organic acids, especially ACA, at initial steps of sourdough propagation is associated with competitiveness between species (De Vuyst et al., 2014) and with the microbial balance, which occurs until the establishment of the dominant microbiota. Afterwards, lower concentrations were found and certain stability was observed, coinciding with the pH stabilization. According to Ventimiglia et al. (2015), the stabilization of these parameters indicates that the sourdough is mature and that the microbial metabolism and dynamics are stabilized. Other studies (Ercolini et al., 2013, Harth et al., 2016; Van der Meulen et al.,

2007; Vrancken et al., 2011) have confirmed the stabilization of the microbial community between seven and ten days of propagation, despite some slight variations, confirmed by the stabilization of technology parameters such as acidification and ratio between LAB and yeasts.

In our work, the temperature did not influence the balance between LAB and yeast between the two sourdoughs but influenced the acidification kinetics, since SD2 presented higher acidifying capacity. Differences in pH and TTA between SD1 and SD2 may reflect differences in microbial community dynamics. Substantial effect on modulation of microbiome and its activity can be attributed to the temperature of incubation, related to the ability of microorganisms to adapt to low or high temperatures and acid conditions (Vrancken et al., 2011). On the other hand, different environmental conditions have proven to influence kinetics more significantly than in the composition of the dominant microbiota (Van Kerrebroeck et al., 2016). Temperature of incubation also had influence on synthesis of organic acids. SD1 presented the highest concentration of ACA, while the highest concentrations of CTA and LCA were found for SD2. Metabolites produced during fermentation can be correlated to temperature and predominance of hetero- or homofermentative species in sourdough. Heterofermentative LAB are able to metabolize carbohydrates into a mixture of LCA and ACA (Harth, Van Kerrebroeck, & De Vuyst, 2016). Besides that, high concentrations of ACA and other volatile metabolites are typical in sourdoughs fermented at 30 °C and dominated by heterofermentative LAB and yeasts (Weckx et al., 2010). Thus, the temperature can affect the dynamics of the microbial community, modifying the species (Vrancken et al., 2011) or just changing metabolic patterns (Van Kerrebroeck et al., 2016).

SC doughs presented lower concentrations of organic acids, therefore, not even prolonged fermentation using baker's yeast was able to produce LCA and CTA as much as SD. After baking, SD breads maintained the concentrations of organic acids higher than SC breads, however, lower in relation to the doughs due to volatilization of these metabolites at high temperatures (Pétel, Onno, & Prost, 2017). The amount of acids retained for the sourdough breads contributes to flavor, texture, digestibility and shelf life (Bartkiene et al., 2017; Dagnas et al., 2015; Gobbetti et al., 2014; Pétel, Onno, & Prost, 2017). Acidification also influences fructans degradation, by the activation of invertases

(Nilsson, Öste, & Jägerstad, 1987). Higher concentration of organic acids in SD breads is important for the degradation of FODMAPs.

The content of short-chain carbohydrates for wheat flour is consistent with already described results by other researchers (Shewry & Hey, 2015; Struyf et al., 2016; Ziegler et al., 2016). The wheat variety can affect the levels of FODMAPs, as well as growing locations, season, climate, cooking methods, and storage time/temperature of product (Muir et al., 2009; Tuck et al., 2014; Ziegler et al., 2016). These findings are potentially important, since the wheat, and consequently the breads, have been shown to contain a number of different types of short chain carbohydrates. Concentration of FODMAPs were obtained by the sum of the isomers (SOR + MAN; GLU + FRU) or saccharides with similar structures (NYS + STA; KES + RAF), since it was not possible to separate them under the chromatographic tested conditions. Its quantification in subgroups based on their similarity is important because each type is absorbed differently in the digestive tract.

SUC, GLU and FRU were totally consumed already from BS1. This rapid decrease corresponds to the intense metabolic activity of microbiota and consequent conversion of these carbohydrates into organic acids, confirmed by the higher concentration of acids produced during the first steps of propagation. According to Struyf et al. (2016), SUC, GLU and FRU are degraded very fast during mixing and the first hours of wheat dough fermentations, due to the invertase activity of microorganisms and amylase activity of wheat. At dough stage, flour SUC is rapidly cleaved into GLU and FRU. GLU is used as energy source, whereas the use of FRU results in excretion of MAN and acetate (Gobbetti et al., 2005), evidenced by the high production of acetic acid and MAN at the first steps of the propagation.

Although SUC is not classically included in the FODMAPS group, a recent study (Henström et al., 2018) demonstrated the implication of SUC in triggering IBS symptoms for individuals with a rare variation in the SUC-isomaltase gene, a condition that reduces the digestibility of SUC. Therefore, for individuals with IBS, the amount of SUC becomes important as well. For *S. cerevisiae*, the metabolism of SUC begins with its hydrolysis by the extracellular invertase. Subsequently, monosaccharides (GLU and FRU) are transported into the cells by facilitated diffusion and become available for intracellular phosphorylation by gluco- and hexokinase (Marques et al., 2016). SUC metabolism in LAB is mediated by sucrose phosphorylase or sucrose-1-phosphate hydrolase. FRU is utilized as a carbon source by

homofermentative LAB but used as an electron acceptor for the regeneration of reduced cofactors by most heterofermentative lactobacilli.

The more complex subgroups of FODMAPs (GOS, FOS and fructans) are consumed from BS3 onward, when higher BAL and yeast counts were observed. These carbohydrates are utilized when the sources of mono- and disaccharides are exhausted and its degradation is a result of the enzymatic activity of the fermentative microbiota and processing conditions of the breads. Some studies in simulated media have confirmed the ability of LAB and yeast relevant to sourdough – such as *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus* and *S. cerevisiae* – to ferment GOS, FOS and fructans (Saulnier et al., 2007; Pan et al., 2009; Kunova et al., 2011). For doughs fermented with *S. cerevisiae*, the fructans undergo partial degradation due to invertase activity present in yeasts (Loponen & Ganzle, 2018). Invertase can also hydrolyse RAF and the polysaccharide inulin (Marques et al., 2016). Most lactobacilli harbor some specific enzymes that possibly play a pivotal role in FODMAPs metabolism (Teixeira, Mcneill, & Ganzle, 2012; Tieking et al., 2005). For instance, α-Gal, an intracellular enzyme that cleaves the terminal non-reducing α-galactose residues from RAF and STA (Silvestroni et al., 2002; Teixeira, Mcneill, & Ganzle, 2012); 1,6-α-glucosidase, capable of hydrolyzing gluco-oligosaccharides (Ganzle & Follador, 2012) and levansucrase and sucrose phosphorylase, able to convert raffinose-type sugars (Tieking et al., 2005). Extracellular LAB enzymes are responsible for the degradation of large fractions of fructans, leading to extracellular accumulation of short fractions, which can be taken up to the bacterial cell for the intracellular hydrolysis (Tsujikawa, Nomoto, & Osawa, 2013). LAB typically found in sourdough are able to cleave and convert fructans to FRU and SUC. In sourdoughs composed by yeasts and bacteria, LAB can further increase the degradation of fructans, by creating acidic conditions that improve the invertase activity of yeasts (Nilsson, Öste, & Jägerstad 1987). This effect may have contributed to the higher fructan degradation observed for sourdough breads, since organic acids concentration was higher for SD doughs. The invertase produced by *S. cerevisiae* is also able to degrade (partially) fructans to GLU and FRU (Menezes et al., 2018), however, this capacity is limited. The hydrolysis of fructans is limited to baker's yeast because degradation of fructans with a degree of polymerization of more than four depends on extracellular fructanases,

which are not expressed by *S. cerevisiae*. However there are some lactobacilli expressing fructanases such as *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus paracasei*, *Lactobacillus pentoses*, *L. casei* and *L. curvatus,* (Loponen and Gänzle, 2018; Paludan-Müller, Gram, Rattray, 2002; Singh, Chauhan, & Kennedy, 2017).

There was no significant difference between the total content of fructans of both SC breads, however, the degradation was slightly higher when the fermentation time for *S. cerevisiae* was increased (SC2). In other study (Fraberger et al., 2018), a strain of *S. cerevisiae* isolated from Austrian traditional sourdough had a degradation potential of 18% within 6 h of incubation, and a decrease of 54% after 72h in the wheat fructans content. Straight doughs are typically obtained upon short fermentation (0.5–3 h), which causes a relatively limited hydrolysis of cereal components, including FODMAPs. On the opposite, sourdough biotechnology requires longer fermentation time. Besides that, sourdough have a great potential for driving FODMAPs concentrations in bread because it often includes both microbial populations, LAB and yeasts (Menezes et al., 2018). The presence of LAB improve the metabolic capacity of the fermentation microbiota, and the long fermentation time allows an increased enzymatic activity on the dough components (Ganzle, 2014). Fructans degradation mainly occurs during mixing and fermentation, although the final baking step may have an impact as well, since fructans are susceptible to degradation during dry heating, and heating in acid conditions (Verspreet et al., 2005). This effect can be observed by the reducing the content of fructans in all treatments after baking, and confirmed by the increase of SUC, FRU and GLU, which are released when the fructans are degraded (Fraberger et al., 2018).

As LAB can convert fructans into FRU, and FRU in turn, can be converted to MAN, MAN levels should also be determined in sourdough breads intended for IBS-sufferers. Unlike the other subgroups of FODMAPs, the amount of polyols increased during sourdough propagation and during fermentation of the SD doughs and, consequently, were significantly ($p < 0.05$) higher for SD breads. Heterofermentative LAB were able to produce MAN using FRU as an alternative external electron acceptor, reducing it into MAN, by MAN dehydrogenase. This pathway represents a competitive advantage in sourdough, because results in extra ATP. Besides that, the excretion of MAN may lead to equilibration of the redox balance (Vrancken et al.,

2011). Higher concentrations of MAN were observed for SD1. Differences in temperature of incubation during the propagation steps may change competitiveness and metabolism of both SD communities. High concentrations of MAN were positively correlated with high ACA concentrations and the presence of certain species, such as *L. fermentum*, *Lactobacillus sanfranciscensis*, *Lactobacillus curvatus* and *Leuconostoc citreum* (De Vuyst et al., 2009; Gan-Erdene et al., 2011), generally observed during the first four or five days of propagation, when a stable ecosystem was still establishing, indicating their contribution to the strains' competitiveness (Wenckx et al., 2010). From technological aspects, synthesis of MAN and ACA have positive effects. Mannitol can influence the sensory characteristics because of its flavor, while ACA contributes with flavor and texture (Arendt, Ryan, & Bello, 2007; Pétel, Onno, & Prost, 2017), and can extend the shelf life of breads, due to antifungal activity (Dagnas et al., 2015). Although there was an increase in the concentration of polyols, which is not ideal for IBS-sufferers, Costabile et al. (2014) showed that sourdough bread is less likely to lead to IBS symptoms compared to conventional bread, despite the higher levels of MAN, an indication that this subgroup of FODMAPs is probably not alone responsible for the triggering of IBS symptoms. While heterofermentative lactobacilli convert FRU to MAN, the degradation of MAN requires MAN-fermenting lactobacilli *(e.g. Lactobacillus delbrueckii, Lactobacillus casei, L. plantarum* and *Lactobacillus salivarius* groups*).* Therefore, combination of MAN-fermenting and fructan-fermenting LAB allows fructans degradation and conversions of MAN, for the production of bread with low content of fructans and MAN (Loponen & Ganzle, 2018).

Despite the increase in SOR+MAN for SD breads, polyols concentrations were very small in bread, being at least 200 times lower than fructans, so their synthesis does not compromise the reduction of the total concentration of FODMAPs promoted by sourdough. The reduction in the total fructans content is the most interesting aspect of sourdough fermentation to IBS patients, because they are the major part of the FODMAPs found in wheat. According to Muir et al. (2019), is not appropriate that the bread had zero FODMAP, just only reduce it to quantities that can be tolerated by IBS- sufferers, since some FODMAPs (fructans, GOS) are prebiotic and therefore is not desirable to completely restrict this carbohydrates in the diet. The majority of people with IBS can tolerate ½-1 slice of many wheat breads, corresponding to less than 300 mg of FODMAPs. SD breads had a concentration of

FODMAPs about 100 to 200 mg/100g of bread, while SC breads had 400 to 500 mg/100g. According to Halmos et al. (2014), the FODMAPs intake would be < 500 mg per meal, considering other sources, in addition to bread. Thus, the SD breads obtained in this study are suitable for IBS- sufferers.

5. FINAL CONSIDERATIONS

The use of sourdough starters affects the total FODMAPs content and the synthesis of organic acids during the fermentation of sourdough and bread making. The sourdoughs investigated presented different potential of acidification, FODMAPs degradation and synthesis, which may be attributed to the temperature difference. The largest reduction of FODMAPs was observed in the sourdough produced at 30 °C. Sourdough fermentation had different implications on FODMAPs. Sourdough breads had less SUC, FRU + GLU and fructans compared to the use of baker's yeast. On the other hand, it presented higher concentration of polyols. Despite this, the reduction of fructans concentration was much more expressive than the synthesis of polyols and SD breads presented lower total content of FODMAPs. SD breads produced were adequate for individuals with IBS, as the total concentration of FODMAPs were lower than the recommended maximum values in the diet. The results of this study demonstrate the potential of sourdough for the production of low FODMAPs bread. Technological modulation of FODMAPs levels during sourdough fermentation are useful for the expansion of tools to producing breads with specific nutritional characteristics, representing a promising approach for IBS and NCGS patients.

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TS 1. Optimized MRM parameters for the determination of FODMAPs, organic acids, NIS, and NAT, in the mass spectrometer [a]

Analyte	RT (min)	Precursor ion (m/z)	Product ion (m/z)	DP(V)	CE (V)
SUC	14.9	-340.9	-179.1	-155	-20
			-119.1		-24
GLU	11.8	-178.8	-88.9	-60	-12
			-100.9	-100	-14
FRU	12.7	-179.1	-88.9	-55	-35
			-143.0	-45	-11
SBL	13.2	-180.9	-101.0	-115	-20
			118.9		-18
MAN	13.2	-180.9	118.9	-65	-16
			101.1		-18
RAF	16.80	-502.8	179.0	-205	-30
			221.1		-36
KES	16.80	-502.9	179.0	-205	-28
			323.2		-36
NYS	17.4	-664.8	179.0	-245	-36
			485.2		-34
STA	17.4	-664.8	383.1	-265	-46
			178.9		-40
LCA	3.41	- 89	-43.3	-34	
			-45.1	-27	
CTA	3.43	193.1	129.0	31	17
			175.0	31	9
BEN	5.85	122.9	79.0	36	17
			51.0	36	51
SOR	5.65	112.9	67.0	56	19
			65.0	56	25
[a]NIS	6.41	672.1	811.3	56	23
			649.3	56	23
NAT	7.62	662.2		56	
				56	

Molecular ion state (-1/+1). [a]Positive ionization state with five charges. RT = Retention time; DP = Declustering potential; CE = Collision energy.

THESIS CONCLUSION

The evolution of the microbial composition of wheat sourdoughs produced at 21°C ± 1ºC (SD1) and at 30 °C ± 1ºC (SD2) and the effects of sourdough fermentation on the content of FODMAPs in breads were evaluated for the first time in Brazil. In our study, wheat flour was not the only source of microorganisms. Lactic acid bacteria (LAB) were just detected from the first propagation step (BS1) onward, possible coming from the environment of production. From BS4, where the production of organic acids was more pronounced, LAB became predominant, being able to inhibit other genera. From BS5 onward, *L. farciminis* became predominant for both sourdoughs; however, the temperature difference between them altered the composition of the respective sub-dominant microbial populations. *L. farciminis* is not usually a predominant microorganism in European sourdoughs, demonstrating that Brazilian sourdough is a potential source of LAB with metabolic characteristics interesting to industry and human nutrition.

At the end of the propagation steps, only LAB were detected in SD1. For SD2, in addition to LAB, the microbial community was composed by other genera. At 30 ºC, the persistence of other bacterial groups as *Enterobacteriaceae* were favored; whereas at 21 ºC an antagonistic relationship between LAB and non-LAB were observed. The temperature variation also altered the metabolic activity of sourdoughs. The sourdough propagated at 30 ºC resulted in breads with higher concentration of acids and lower FODMAPs content. However, due to the presence of non-typical sourdough bacteria, 21 ºC can be considered most suitable for propagation of sourdoughs in Brazil. Temperature proved to be an important factor in shaping the sourdoughs microbial community and for the quality standardization of sourdough bread.

Both sourdough microbial communities were able to promote the reduction of the total content of FODMAPs in wheat bread at levels that may be tolerated by IBS-sufferers (reduction between 40 to 60% for SD1 and SD2, respectively). Sourdough breads presented a significantly lower concentration of fructans, sucrose, fructose and glucose, in comparison with control breads, produced with *S. cerevisiae*. Sourdough fermentation has a great potential to production of low-FODMAPs wheat bread, representing an alternative for IBS- and or NCGS-sufferers, individuals for whom Low-FODMAPs diets are recommended.

FINAL CONSIDERATIONS

For further studies, it is suggested the characterization of the microflora of a greater number of sourdoughs elaborated in Brazilian bakeries, in order to promote the identification of the microbial communities of sourdoughs produced in Brazil. Our research group is working in the isolation and identification of LAB strains obtained from Brazilian sourdoughs and in the characterization of their technological potential for industry and human nutrition and health. Regarding IBS and NCGS conditions, selected strains capable of degrading fructans and polyols are being tested to obtaining lyophilized sourdoughs and used to producing low-FODMAPs bread. This biotechnology has great relevance for the bakery industry and for human nutrition. Finally, we are studying the effects of sourdough fermentation on the protein fraction of wheat, also related to NCGS. Clinical trials with consumers and other studies regarding the applicability of sourdough fermentation to improving nutritional characteristics of breads are strongly encouraged.

We would like to highlight the relevance of the internationalization of Brazilian Post Graduate Programs through the Programa de Doutorado Sanduíche no Exterior (PDSE). Thanks to this Program, an important part of this research could be carried out in an institution of international renown, generating positive results for the student, for the research group, for the University, for Brazil and the world.. We hope that the results of this research will contribute to the strengthening of the recognition not only of the Federal University of Santa Caratina (UFSC) as an institution that produces knowledge and science, but also of the Brazilian Public Universities in general.

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REVIEW
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Effects of Sourdough on FODMAPs in Bread and Potential Outcomes on Irritable Bowel Syndrome Patients and Healthy Subjects

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Background: Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) are an heterogeneous group of compounds that can be poorly digested and may have a range of effects on gastrointestinal processes. FODMAPs are found in a wide variety of foods, including bread. FODMAPs' intake is associated with the onset of symptoms of irritable bowel syndrome (IBS). On the other hand, some FODMAPs contribute to the healthy maintenance of intestinal microbiota. Volume increase of bread dough commonly relies on the use of two biological leavening agents, sourdough and baker's yeast and, in some cases, a combination of both.

Scope and Approach: The main objective of this review is to discuss the association between FODMAPs and IBS, beneficial effects of FODMAPs on healthy subjects and potential impact of biological leavening agents on FODMAPs content of bread.

Key Findings and Conclusion: Given that yeasts and lactic acid bacteria, the dominant microorganisms in sourdough, may degrade FODMAPs, it would be possible to modulate the FODMAPs concentration in bread, thus positively affecting consumers' health.

Keywords: fermentable oligosaccharides, disaccharides, monosaccharides and polyols, irritable bowel syndrome, bread, lactic acid bacteria, yeasts, sourdough

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INTRODUCTION

Nowadays, tailored nutritional recommendations may be designed in order to treat or prevent diseases (Betts and Gonzalez, 2016). For instance, lactose- and gluten-containing foods must be avoided by subjects suffering from hypolactasia and celiac disease (De Toro-Martín et al., 2017). Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) are an heterogeneous group of compounds (most of which are short-chain carbohydrates) that can be poorly digested and may have a range of effects on gastrointestinal processes. This group includes lactose, fructose in excess of glucose, fructans, and fructooligosaccharides (FOS, such as nystose and kestose), galacto-oligosaccharides (GOS such as raffinose and stachyose), and sugar polyols (sorbitol and mannitol) (Muir et al., 2009).

Fermentable oligosaccharides, disaccharides, monosaccharides and polyols are found in a wide variety of foods. Their dietary uptake mostly results from honey and fruits as watermelons, pears, and apples (fructose); milk and dairy products (lactose); rye, wheat, artichoke, garlic, onions,

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ORIGINAL PAPER

Use of sourdough fermentation to reducing FODMAPs in breads

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Abstract
Fermentable, oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) are a class of carbohydrates poorly digested that may trigger the symptoms of Irritable Bowel Syndrome (IBS) and Non-Celiac Gluten Sensitivity (NCGS). The effects of sourdough fermentation on FODMAPs and organic acids were studied during the sourdough propagation and bread making. The concentrations of organic acids were higher for the first steps of propagation and became stable for final steps. All FODMAPs were significantly reduced during the propagation, except polyols. Sucrose, fructose and glucose were wholly degraded for the first step of fermentation. The other carbohydrates had their concentrations reduced after the fourth backslopping step. Sourdough bread presented the higher level of organic acids and polyols, and lower content of fructans, sucrose, fructose and glucose than bread fermented by *Saccharomyces cerevisiae*. The fructan reduction was from 69 to 75%, indicating that sourdough fermentation can be applied for producing low-FODMAP wheat bakery products.

Keywords Short-chain carbohydrates · Irritable Bowel Syndrome · Sourdough bread · Fructans · Non-celiac Gluten Sensitivity · Lactobacilli

Introduction

Sourdough results from the mixture of cereal flour and water, fermented mainly by lactic acid bacteria (LAB) and yeasts, which can grow spontaneously, from the flour and other ingredients, or can be added as a starter culture [1, 2]. Several studies have confirmed the ability of sourdough fermentation to promote beneficial modifications on sensory [3], technological and nutritional properties [1, 4, 5] of leavened baked goods. These effects are, however, dependent on the microbial composition of sourdough [6], which is modulated by some parameters as time and temperature of fermentation, and type and origin of flour [2].

The more expressive metabolic activities of microbial communities during fermentation are acidification, leavening, and flavor formation [7], all related to metabolism of carbohydrates. Conversions involving hexoses and pentoses are well explored in the literature [8]; however, fermentation of other saccharides remains little known. Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) are an heterogeneous class of compounds (most of which are short-chain carbohydrates) that includes lactose, fructose in excess of glucose, fructans and fructooligosaccharides (FOS, nystose, kestose), raffinose family of oligosaccharides (GOS, such as raffinose and stachyose),

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SUPPLEMENT C – CERTIFICATE OF ORAL PRESENTATION IN IV INTERNATIONAL CONFERENCE ON MICROBIAL DIVERSITY – BARY, ITALY.

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