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Investigating the biodiversity in spontaneous fermentations as a source of high-performance microorganisms

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1. ABSTRACT

This PhD project was aimed at the investigation of spontaneously fermented food products with the goal of selecting highly performing strains to be used as starter cultures, in order to optimize the fermentation process and give to the final product an added value from a technologic, safety or sensory point of view.

This project is divided in two parts dealing with two different fermentation processes. The first part is focused on sourdough fermentation and its potential to improve the baking performance of alternative grains such as spelt. Firstly, the dominating lactic acid bacteria (LAB) and yeasts were isolated and studied for their performance in fermentation as well as resistance to stress conditions. From the selected strains, one strain of *Pediococcus pentosaceus* was selected as a potential starter.

To design a mixed starter culture, other LAB and yeast strains isolated from cereal fermentations were studied. First, we studied two LAB species, *Weissella cibaria* and *W. confusa*, that have interesting pro-technologic characteristics such as a good ability to produce exopolysaccharides, to degrade arabinoxylans and to inhibit the growth of some mold species. On the other hand, the two species have raised concerns as potential opportunistic pathogens. For this reason, combined genotypic and phenotypic studies were performed to assess the safety of the two species.

Subsequently, an alternative non-*Saccharomyces* yeast species, *Kazachstania unispora,* was investigated as a leavening agent. The species had a good resistance to stress conditions and a good ability to ferment glucose, but lacked the ability to utilize maltose, the main carbon source in dough, for this reason it performed a slow leavening depending on the LAB to release free glucose.

The selected strains were applied to a spelt-based sourdough bread with promising results; the LAB increased the mold-free shelf-life and improved the

texture and conservation of the product, that received a high liking score in the sensory evaluation.

The second part of the project regarded the cocoa fermentation and the possibility of using selected LAB as adjunct cultures to improve the quality of the chocolate in a controlled fermentation setup. Firstly, LAB and yeast strains were isolated and identified from natural cocoa fermentations. Two strains belonging to the species *Lactiplantibacillus fabifermentans* and *Furfurilactibacillus rossiae* were selected for further studies. We evaluated their adaptation to conditions associated with cocoa fermentations, such as low pH, high temperatures and high osmotic pressure as well as their ability to grow in conjunction with yeasts and acetic acid bacteria, whose activity is essential for cocoa fermentation. The two strains were then used as adjunct cultures in cocoa fermentations and the quality and sensory profile of the chocolate obtained was evaluated.

This work allowed us to highlight the potential of autochthonous microbial strains with high level adaptation to the food matrix that allow not only to obtain a successful and reliable fermentation but to add to the safety, taste and quality of the final product.

2. INTRODUCTION

Fermentation has been used as a way of food preservation for millennia, with reports dating as early as 6000 BC (Salque et al., 2012). Although this process was conducted in an uncontrolled manner and without the complete understanding of the underlying process or of all the benefits that fermentation conferred to the food products, fermentations got embedded in the culture and traditions of populations throughout the world.

Whereas initially fermentations were spontaneous, hard to predict and probably with variable results, eventually the advantages of conserving small quantities of a successful fermentation to add to the new fermenting mass, also known as backslopping, were discovered (Ravyts et al., 2012).

With the industrial revolution of the 19th century, the dynamics of the populations changed; more and more people were living in urban areas, which required food manufacturing to move away from the artisanal manner of production, and to maximise the reliability and productivity (Caplice and Fitzgerald, 1999). In this period the science of microbiology started to bloom, from the experiments of Louis Pasteur in 1859 that denied the theory of "spontaneous generation" to the work of Sir John Lister in 1877 that showed the role of a single bacterium, "*Bacterium lactis*", or as it is known nowadays, *Lactococcus lactis*, in milk fermentation (Bourdichon et al., 2012). These particular circumstances propelled by technology advances in food production led to the selection of microorganisms to be used as fermentation starters, that were further studied and applied throughout the 20th century.

Nowadays, starter cultures are available for many fermented foods products, that guarantee consistency and reliability, but spontaneous fermentations and backslopping are still used in some industries, such as in cocoa fermentation, as well at a small artisanal level. Due to the different flavour profiles that spontaneous fermentations confer to the products when compared to the standardized industrial starters, very often these products are considered to be premium and are very much appreciated and requested by consumers. But

the only way to meet this increasing demand is to move the production to an industrial level, thus, to use new starter cultures, that in return decrease the products' variability and uniqueness. It becomes thus a new challenge for food scientists to discover ways of conducting large scale fermentations at the highest quality while preserving the typical flavours of the original fermented food products.

While initially it was developed as a conservation method, fermentation confers many positive characteristics to food products. Some of the beneficial roles of fermented foods are:

- Food preservation and safety.
- Improved sensory properties.
- Improved structure and consistency.
- Improved digestibility.
- Superior nutritional properties.
- Degradation of antinutritive compounds.
- Probiotic and prebiotic effect (Ravyts et al., 2012).

Many of these beneficial traits are related to the fermentation performed by lactic acid bacteria (LAB). Their ability to lower the pH of the food to safe levels, under which spoilage and pathogenic microorganisms are inhibited is a direct result of fermentation performed by these bacteria, that can be either homo- or heterofermentative. The production of acetic acid in heterofermentation increases the inhibitory activity of the organic acids, towards yeasts, fungi and Gram-negative bacteria. LAB have also been known to produce bacteriocins, that inhibit the growth of Gram-positive bacteria as well as pathogens, acting as biocontrol agents and reducing the need of artificial preservatives. The production of organic acids as well as other metabolites by LAB impact the flavour of fermented food products. LAB found in fermented foods may survive the gastro-intestinal tract transit and have an impact on the gut microbiota, whereas exopolysaccharides produced in some fermented products may act as prebiotics (Leroy and DeVuyst, 2004).

The main role of yeasts in food fermentations is closely related to their ability to conduct alcoholic fermentation, with ethanol and CO_2 being the main metabolites. These metabolites are essential in the production of alcoholic beverages, as well as dough leavening. Yeasts have also the ability to produce other organic acids, aldehydes, ketones, higher alcohols that have an impact on the taste, flavour and texture of fermented foods. Furthermore, they produce a number of hydrolytic enzymes such as proteases, lipases, amylases, invertase, xylanases, pectinases, phytases, that play an important role in the production or quality improvement of fermented foods. Their ability to improve the bioavailability of vitamins and minerals as well as the digestibility of proteins enhances the nutritional value of products (Rai and Jeyaram, 2017).

Other microorganisms that conduct food fermentations are acetic acid bacteria that are essential in products such as vinegar and cocoa, coagulase-negative staphylococci are found in fermented meat products, brevibacteria, corynebacteria and propionibacteria are used in cheese production whereas *Bacillus subtilis* is essential for soybean fermentation. Molds are also used in some fermented products such as in blue cheeses, sausages, miso, soy sauce and tempeh (Ravyts et al., 2012).

In order to be used in starter cultures, microbial strains should be safe to use and not pose threats to the health of consumers. For this reason, food regulatory agencies have set up approved lists of microbial species that are safe for human consumption and industrial utilization. In the United States of America, the Food and Drug Administration (FDA) agency includes microbial strains together with food additives in the Generally Recognised as Safe (GRAS) list, that bases the evaluation on available scientific data as well as experience of use of the ingredient. In the European Union, the European Food Safety Authority (EFSA) includes microbial species authorised for commercial use in the Qualified Presumption of Safety (QPS) list, clearly defining the workflow for the approval of new microorganisms, that periodically get added to the list. The QPS lists contain microorganisms that do not raise

concern for human health, evaluated considering several factors, such as a clear taxonomic evaluation, the current body of knowledge, the proved absence of safety concerns as well as the clear definition of its intended use. Microorganisms included in the QPS list do not need to undergo a full safety evaluation before their use, and their safety is guaranteed by continuous reviews of the available scientific literature (FEEDAP et al., 2018)

In addition to these lists, the International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (EFFCA) suggested an alternative Inventory of Microbial Food Cultures Demonstrated to be Safe, that considers species used mainly in milk fermentation based on the available literature and attempts to be an exhaustive list, in contrast to the QPS list that only considers species that have been submitted for approval (Bourdichon et al., 2019).

There are several safety concerns that need to be elucidated for microbial strains before their use at industrial scale. One of the most important problematics that is gaining increasing attention from the scientific community is the resistance to antimicrobials, in particular to antibiotics. For this reason, The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has defined cut-off concentration values for antibiotics that strains intended for industrial use should not exceed. Furthermore, the absence of transferable resistance genes should be considered for a safe use. Other safety factors that need to be elucidated are the absence of pathogenicity and virulence traits and production of toxins or biogenic amines.

Considering all these observations regarding the design and application of starter cultures, the aim of this PhD thesis was to assess the enzymatic and metabolic activities of different LAB and yeast strains, to select the most promising ones to set up new starter or adjunct cultures and to apply them to food fermentations in order to assess their overall quality.

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RESULTS

3. Characterization of microbial populations of fermented cereals and related microbial strains for their use as starter cultures

3.1 STATE OF THE ART

Cereals are one of the most inexpensive and widely used sources of energy and nutrients in the world. Although the production of a variety of cereal-based foods, such as biscuits, pasta or boiled rice do not require fermentation, many products are obtained by either alcoholic fermentation, lactic acid fermentation or a combination of both.

Sourdough is a prime example where a mixed alcoholic and lactic acid fermentation occurs. This is thought to be one of the first uses of microbial process in human history and can be traced back to 1500BC (Hammes & Ganzle, 1998). The main technological function of sourdough is leavening, but this fermentation process imparts to the products several other desirable characteristics.

Firstly, sourdough fermentation modifies the structure of the dough by partial hydrolysis of starch and proteins. This ability is especially relevant in products obtained with grains that have low baking performance, such as rye bread, by solubilizing pentosans and increasing their ability to retain water and gas. Furthermore, exopolysaccharides synthetised by the LAB are an additional source of hydrocolloids that improve the texture and volume of sourdough products (Arendt et al., 2007). In this context, another important activity of sourdough is the inhibition of excessive activity of endogenous α-amylases, that are especially active in rye flour and that may completely hydrolyse starch during the baking process. Sourdough fermentation inhibits these enzymes by lowering the pH of the dough (Decock & Cappelle, 2005).

Another important role of sourdough in baked products is the production of flavour precursors and flavour components that give the final product a characteristic and very desirable taste and flavour profile. The most important molecules for the typical sourdough bread taste are lactic and acetic acids, but other flavour compounds have been found to play a relevant role. The conversion of arginine to ornithine through the arginine deiminase pathway is one of the most impactful microbial activities that on the crust flavour during

baking (Pétel et al., 2017). In addition, the release of free amino acids either directly improve the taste of the bread, as is the case of glutamate, or act as precursors to flavour compounds: ornithine, leucine and phenylalanine are converted to 2-acetyl-pyrroline, 3-methyl-butanol and 2-phenylethanol, respectively (Xu et al., 2019).

Sourdough based products are also much more resistant to microbial spoilage, either mould growth or rope-forming bacilli (Menteş et al., 2007). This has a positive impact on the shelf life and safety of these products. The possibility of substituting preservatives with sourdough fermentation maintaining a clean label is very desirable for the consumers, that generally prefer to avoid products containing additives. The main metabolite with antimicrobial activity is acetic acid, but other metabolites with specific antibacterial or antifungal activities have been identified in sourdough. Phenyllactate and hydroxy phenyllactate produced by *Lactiplantibacillus plantarum* have been proposed and applied as antimould compounds in sourdough bread (Lavermicocca et al., 2003; Quattrini et al., 2016). The production of such compounds is strain specific to LAB, so a targeted selection of the strains to be used is necessary to maximise the positive effect on the mould free shelf life.

Sourdough fermentation also improves the nutritional quality of bread, either by degrading antinutritional compounds such as phytic acid, or by producing molecules with health benefits, such as γ-aminobutyric acid and bioactive peptides. The phytate degradation is mainly associated with the increased solubility due to the low pH and the action of endogenous phytate degrading enzymes, although there have been studied several yeast strains that have the ability to synthesize phytases, which can further degrade this antinutritional compound (Gobbetti et al., 2019).

Finally, sourdough-based products have been described to have a lower glycemic index when compared to the straight dough method. One possible reason for this characteristic is the different interaction between starch and gluten in presence of lactic acid (Fardet et al., 2006). Furthermore,

exopolysaccharides produced by LAB may also have prebiotic characteristics (Galle & Arendt, 2014).

Besides the positive impact of sourdough fermentation on the properties of baked products, this is also embedded in the culture and traditions of many countries. For this reason, there is a high demand by the consumers that the industry needs to meet by applying new technologies to successfully supply a high-quality product in a standardized manner.

From an industrial point of view, there are three main types of sourdough:

- Type I sourdough is one obtained by the previous fermentation through backslopping, that is, by using pre fermented dough as inoculum for subsequent fermentations. This is the traditional way of sourdough production.
- Type II sourdough is obtained with the addition of *Saccharomyces cerevisiae* as well as other selected strains.
- Type III sourdough is a type II sourdough subjected to drying to increase its stability (Decock & Cappelle, 2005).

The microbial composition of these types of sourdough varies in function of the fermentation parameters and the characteristics of flour used, but the communities are very stable, indicating a high level of adaptation to these specific conditions.

Type I sourdough is characterized by the presence of *Fructilactobacillus sanfranciscensis* as dominant species (Gänzle & Zheng, 2019). This obligate heterofermentative lactobacillus is highly adapted to sourdough and develops rapidly in sourdough-related conditions such as low pH and room temperature and has a mutualistic relationship with *Candida humilis* (Brandt et al., 2004). Other species of *Lactobacillus, Weissella, Leuconostoc* and *Enterococcus* are also found in sourdough. The main yeast species encountered are *C. humilis* and *Saccharomyces exiguus*, as well as *S. cerevisiae.*

Type II sourdough fermentation parameters differ from those of type I sourdough for the higher fermentation temperature and more extended fermentation time, thus a higher acidity. These conditions are more favourable

for the growth of homofermentative lactobacilli and pediococci, as well as acid tolerant and thermophilic LAB such as *L. reuteri* (Zheng et al., 2015).

Sourdough fermentation is a complex and dynamic process, the applicability and reproducibility of which can be problematic at industrial level. For this reason, the selection of specific strains that can be inoculated as starter cultures is preferred. Furthermore, strains can be selected not only for their robustness, but also for specific enzymatic and metabolic activities that improve the characteristics of the final product for one or more of the aforementioned parameters.

Considering the positive effects of sourdough on the texture and flavour of baked products, there is an increased interest in applying this method to alternative grains that are receiving the attention of consumers and the industry for their sustainability, flavour, and nutritional profile. Spelt is an ancient grain that is being rediscovered for these beneficial characteristics but represents a challenge for the industry due to the low performance in baking. In order to standardize and facilitate its use, the application of a starter culture can be investigated as a natural solution that does not require the use of additives and dough conditioners.

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3.2 AIMS AND OBJECTIVES

In light of the high interest of both consumers and the industry for sourdough-based products, the problematics related to the complexity of the fermentation process and the reduced reproducibility of spontaneous fermentations, as well as the potential benefits of the application of specifically selected strains, the aims of this chapter were to describe the autochthonous microbial population of fermented spelt, to select robust strains from various fermented cereals and explore enzymatic activities of interest as well as the safety of application, using a combined phenotypic and genotypic approach. The selected LAB and yeast strains were applied in a spelt based bread model, and the shelf-life, texture and consumer preference were investigated. Thus, the main objective of this chapter was to design a stable, robust and effective starter culture that could be applied to improve the characteristics and acceptance of spelt based bread.

3.3 Microbial consortia involved in fermented spelt sourdoughs: dynamics and characterization of yeasts and Lactic Acid Bacteria

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Running headline: Spelt sourdough microbiota

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3.3.1 Significance and Impact of the Study: Nowadays, there is a renewed interest in products based on spelt. This “ancient grain" is a highly nutritional grain, however, its use is limited to bread making processes not yet standardized. The low baking and sensory quality of spelt can be overcome through fermentation processes. However, the autochthonous microbiota of spelt sourdough is poorly known. This study highlights the dynamics of microbial communities involved in sourdough fermentation of spelt and provides the basis for the selection of autochthonous cultures, with the aim of improving the nutritional potential of spelt and its rheology and breadmaking properties.

3.3.2 Abstract

This study aims to describe the native microbiota of fermented spelt, taking into consideration both lactic acid bacteria (LAB) and yeasts, for which little data are available. Five samples of commercial spelt flour were subjected to spontaneous fermentation to obtain a type I sourdough. A total of 186 LAB and 174 yeast strains were selected at different refreshment steps and subjected to further analyses. Within LAB, coccal isolates constituted 78.5% of the total LAB strains, with the dominance of *Pediococcus pentosaceus*. Although documented before as a component, this is the first report of a spelt sourdough fermentation dominated by this homofermentative LAB, characterized by a high acidification rate, ability to utilize a wide range of carbon sources and to grow in high osmolarity condition. Yeast communities resulted in four dominant species, *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Pichia fermentans* and *Clavispora lusitaniae.* This study highlights for the first time the biodiversity and dynamics of yeast communities involved in sourdough fermentation of spelt. Compared to commercial baker's yeast, autochthonous *W. anomalus*, *P. fermentans* and *S. cerevisiae* strains show a good performance, and their use could be an advantage for their acquired adaptation to the environment, providing stability to the fermentation process.

3.3.3 Introduction

Cereals represent a staple component of the human diet, and grains such as wheat, rice and, in developing or emerging countries sorghum, maize and millets, provide more than half of the total world calorie intake (FAO 2012). The cultivation of these grains saw an important increase due to their high yield, but current trends are going towards the rediscovery of “ancient grains", such as spelt (*Triticum spelta*), mainly for the increased interest in locally grown crops with particular nutritional properties (Troccoli *et al.* 2005; Frakolaki *et al.* 2018). Particularly, spelt seem to be popular and largely cultivated in Germany, Belgium, Austria, Slovenia and Northern Italy. Despite a lower grain yield, spelt shows an increased resistance to harsh and unsuitable conditions. Indeed, this species can grow in marginal lands, with low soil and climatic requirements, and is suitable for growing without the use of pesticides, making it adequate for the expanding organic food market (Troccoli *et al.* 2005). From a nutritional point of view, spelt has a higher protein content than the standard cultivars of wheat, a higher content of soluble dietary fibre, a high vitamin, minerals and unsaturated fatty acid content, as well as a lower phytic acid content (Bonafaccia *et al.* 2000; Kohajdová and Karovičová 2008). However, its use is still limited in bread making processes, mainly due to the low baking and sensory quality. These limitations can be overcome through a sourdough fermentation process, where a specific microbiota, deriving from natural flour contamination and the environment, interacts for the processing of the matrix in a high-quality product. During sourdough fermentation, a succession of naturally occurring microorganisms results in a population dominated by lactic acid bacteria (LAB) and yeasts. They play a key role for their multiple “functional” metabolic properties (Ganzle 2009).

The biodiversity of the natural microbial population in traditional sourdoughs represents an interesting way for the selection of novel types of cultures, well adapted to the specific fermentation process and able to give a satisfactory

performance in the process. This search requires a screening of a large number of isolates in small-scale fermentations, through which it is possible to highlight the dominant microbial strains characterizing the sourdough and responsible for the final product.

Despite the renewed interest in products based on spelt, sourdough fermentation process and the autochthonous microbiota of this 'ancient grain' are poorly known. The literature refers to a few works, mainly focusing on LAB (Van der Meulen *et al.* 2007; Coda *et al.* 2010). No data are available up to now on the autochthonous yeasts characterizing spelt flour and their interaction with autochthonous LAB species.

This study aims to describe the native microbiota of spontaneously fermented spelt, taking into consideration both lactic acid bacteria (LAB) and yeasts. The dominating strains were identified and characterized.

3.3.4 Materials and methods

3.3.4.1 Sourdough preparation and microbial population

Five samples (A, B, C, D and E) of commercial wholemeal spelt flour of different brands, produced in different Italian regions (Marche, Umbria, Puglia, Toscana and Veneto respectively) were subjected to spontaneous fermentation to obtain a type I sourdough preparation (Manini *et al.* 2014). This type of sourdough is characterized by daily back-sloppings performed by using the previous sourdough to inoculate a fresh water-flour mixture, that helps to maintain the microorganisms in an active state. The fermentation was carried out at 30°C during a period of 7 days. The pH values were recorded daily. Samples at different refreshment steps (2, 4, 5 and 7) were diluted and plated for the determination of: non-lactic acid bacteria (NLAB) on Plate Count Agar (PCA) (Sigma, St Louis, MO, USA), LAB on MRS agar (Difco Lab., Augsburg, Germany) and M17 agar (Difco), yeasts and moulds on Yeast Extract Glucose Chloramphenicol Agar (YGC) (Sigma). The plates were incubated at 30°C for 48 h. To characterize the autochthonous LAB and yeast population, from each flour sample about 10 colonies of LAB and 10 colonies of yeasts were recovered at the refreshment steps 2, 4, 5 and 7 and purified by successive streaking. A total of 186 LAB and 174 yeast strains were selected and subjected to identification and further analyses. A commercial baker's yeast was used for phenotypic comparison. Strains were routinely grown in MRS broth for LAB and Yeast extract Peptone Dextrose broth (YPD) (Sigma) for yeasts.

3.3.4.2 Molecular identification

Total bacterial DNA was extracted from 100 µL of an overnight culture, using the Microlysis kit (Labogen, Rho, Italy) following the manufacturer's instructions. For yeasts, DNA extraction was performed in a PRECELLYS®24-DUAL lyser/homogeniser (Bertin-technologies, Saint Quentin en Yvelines, France), as previously described (Decimo *et al.* 2017).

For LAB identification, a first clustering step was obtained by a PCR amplification of the *16S–23S* rDNA spacer region (RSA). Molecular identification of LAB isolates with different RSA patterns was carried out using species-specific probes and/or by *16S* rDNA gene sequencing. Molecular identification of yeast strains was carried out by a PCR amplification of the Internal Transcribed Spacer (ITS), species-specific probes and/or partial *26S* rDNA gene sequencing. The list of primers and relative thermal cycles is reported in Table 1. Amplification was carried out in a Mastercycler (Eppendorf, Hamburg, Germany). PCR reactions were performed as described before (Decimo *et al.* 2017). Amplicons were purified using NucleoSpin® Extract II (Macherey-Nagel, Düren, Germany) and sequenced at Eurofins Genomics (Ebersberg, Germany). Sequence alignment was carried out with ClustalW software. The NCBI BLAST software was used for sequence similarity search (www.ncbi.nlm.nih.gov/BLAST).

3.3.4.3 Sourdough fermentation-related characterization

To investigate the physiological adaptation of the yeast and LAB community to the sourdough fermentation environment, several characteristics were determined for a subset of strains: utilization of different carbon sources, osmoadaptation, low pH tolerance, and the ability to grow in the presence of acetic acid. For LAB strains autolysis degree and acidification ability were also evaluated. Tests were carried out in duplicate. For carbon source utilization test, YP and a LAB-basal medium (containing g l^{-1}: peptone 15, yeast extract 6, Tween 80 1 ml l^{-1}, pH 6.4) or basal MRS were used for yeasts and LAB respectively. The filter-sterilized carbohydrates were added at a final concentration of 10 g l^{-1} and, for evaluating the osmoadaptation of the strains, at a final concentration of 300 g l^{-1}. To test low pH tolerance, the growth media were adjusted to pH 2.5 and 3.5 with 1 m l^{-1} HCl. To test the ability to grow in the presence of acetic acid, growth media were supplemented with 10 g l^{-1} of acetic acid, after which the pH was corrected to 5.0. All tests were carried out using 1% inoculum of fresh cultures. Growth was determined by

measurements of OD_{600} after 24h of incubation at 30°C. Autolytic phenotype was tested according to Mora *et al.* (2003). The strains were incubated in MRS medium and cells were harvested during the exponential growth phase (OD_{600} between 0.8 and 1), washed in potassium phosphate buffer (50 mM, pH 6.5) and resuspended in the same buffer at pH 6.5 or 4, to and OD_{600} of 0.6 to 0.8. The suspension was incubated at 30°C and the degree of autolysis was expressed as the percentage decrease of the OD_{600} after 48 h.

Table 1 Primer sequences and relative thermal cycles used for the identification of the isolates

Target	Thermal cycle (× 35 cycles)	Sequence (5'-3')	Reference
16S rDNA gene	94 °C × 45 s; 55 °C × 45 s 72 °C × 1 min	F: AGAGTTTGATCCTGGCTCAG R: CTACGGCTACCTTGTTACGA	Lane (1991)
Lact plantarum	94 °C × 2 min; 56 °C × 1 min 72 °C × 1 min	F: CCGTTTATGCGGAACACC R: TCGGGATTACCAAACATCAC	Torriani, et al. (2001)
Pediococcus spp.	94 °C × 45 s; 64 °C × 1 min 72 °C × 1 min	F: GAACTCGTGTACGTTGAAAAGTGCTGA R: GCGTCCCTCCATTGTTCAAACAAG	Pfannebecker and Fröhlich (2008)
Ped. pentosaceus	94 °C × 1 min; 67 °C × 1 min 72 °C × 1 min	F: CCAGGTTGAAGGTGCAGTAAAAT R: CTGTCTCGCAGTCAAGCTC	Pfannebecker and Fröhlich (2008)
Lact. brevis	94 °C × 45 s; 48 °C × 45 s 72 °C × 1 min	F: TGTACACACCGCCCGTC R: TAATGATGACCTTGCGGTC	Coton et al. (2008)
Lact. fermentum	94 °C × 45 s; 48 °C × 45 s 72 °C × 1 min	F: TGTACACACCGCCCGTC R: TTTTCTTGATTTTATTAG	Coton *et al.* (2008)
Lact. paraplantarum	94 °C × 45 s; 56 °C × 1 min 72 °C × 1 min	F: GTCACAGGCATTACGAAAAC R: TCGGGATTACCAAACATCAC	Torriani *et al.* (2001)
Lact. pentosus	94 °C × 45 s; 56 °C × 1 min 72 °C × 1 min	F: CAGTGGCGCGGTTGATAT R: TCGGGATTACCAAACATCAC	Torriani *et al*. (2001)
Lact. rhamnosus	94 °C × 45 s; 54 °C × 45 s 72 °C × 1 min	F: CCCACTGCTGCCTCCCGTAGGAGT R: TGCATCTTGATTTAATTTTG	Ward and Timmins (1999)
Lact.curvatus	94 °C × 45 s; 56 °C × 1 min 72 °C × 1 min	F: GCTGGATCACCTCCTTTC R: TTGGTACTATTTAATTCTTAG	Berthier and Ehrlich (1998)
Leuconostoc spp.	94°C x 45 s; 55°C x 45 s 72 °C × 1 min	F: CCACAGCGAAAGGTGCTTGCAC R: GATCCATCTCTAGGTGACGCCG	Yost and Nattress (2000)
Weissella spp.	94 °C × 45 s; 54 °C × 1 min 72 °C × 1 min	F: CGTGGGAAACCTACCTCTTA R: CCCTCAAACATCTAGCAC	Jang et al. (2002)
ITS1-5.8S-ITS2 internal transcribed spacer (ITS)	94 °C × 45 s; 60 °C × 1 min 72 °C × 1 min	F: TCCGTAGGTGAACCTGCGG R: TCCTCCGCTTATTGATATGC	Jespersen et al. (2005)
26S rDNA gene	94 °C × 2 min;52 °C × 1 min 72 °C × 1 min	F: GCATATCAATAAGCGGAGGAAAAG R: GGTCCGTGTTTCAAGACGG	Kurtzman and Robnett (1998)
S. cerevisiae	94 °C × 45 s; 61 °C × 1 min 72 °C × 1 min	F: GTTAGATCCCAGGCGTAGAACAG R: GCGAGTACTGGACCAAATCTTATG	de Melo Pereira et al. (2010)

3.3.5 Results and Discussion

3.3.5.1 Microbial population of spelt sourdough

Fermentation was characterized by a rapid increase of LAB within the first days of propagation. The number of LAB increased from 10^4 -10^5 CFU g^{-1} of the initial dough, going up to 10^9 CFU g^{-1} after two days of fermentation. This value persisted during all refreshment steps, with little variations. For yeast counts, more variation was observed. Yeasts generally started in a much lower concentration than LAB and reached high amounts (10^6-10^7 CFU g^{-1}), only after 4-5 days of back-slopping. Moulds reached between 3 and 4 logarithmic cycles; in the first steps, but they were completely absent starting from the 5th refreshment, probably due to the antimould activity of LAB and/or yeast communities. In sample B, no mould growth was observed. Viable cell counts performed on different refreshment steps are reported in Table 2.

The pH values did not differ during fermentation for all sourdoughs. The initial pH value resulted to be 6.1±0.27; after 2 days of fermentation an important decrease of the pH was observed, reaching a value of 3.87±0.17, which was maintained until the end of the fermentation (3.64±0.06).

3.3.5.2 Molecular identification

Within LAB, coccal isolates constituted 78.5% of the total LAB strains, characterizing the spelt sourdough analyzed. The identification approach allowed to classify the 146 coccal LAB into three different genera, namely *Pediococcus*, *Leuconostoc* and *Weissella*. The first step, the RSA analysis, highlighted 3 different clusters, one of which included the majority of strains (140). This major cluster was characterized by an RSA profile associated to *Pediococcus* genus (310, 480, 500 bp), the other two clusters showed an RSA profile typical of *Leuconostoc* (600 bp) and *Weissella* (450, 550, 650 bp) genus (Kabadjova et al., 2002). The results were confirmed by genus-specific PCR experiments. The strains characterizing the major cluster were further identified at the species-level. Species-specific PCR confirmed 136 strains to be *Ped. pentosaceus*, and 4 strains were ascribed to *Ped. acidilactici* species.

The RSA profiles of the 40 lactobacilli strains resulted to be poorly discriminating, for this reason a series of species-specific PCRs and *16S* rRNA gene sequencing were carried out. This approach allowed the identification of 18 *Lactobacillus brevis*, 11 *Lact. curvatus* 1 *Lact. fermentum,* 1 *Lact. rhamnosus*. 6 *Lact. plantarum*, 1 *Lact. paraplantarum* and 2 strains of *Lact. pentosus*.

Table 2 Microbial population in spelt flour samples at various refreshment steps. Microbial counts are expressed as log CFU g^{-1}. Values are the means ±standard deviation from two independent experiments.

		Viable cell count (logCFU g^{-1})				
Spelt flour samples	Refreshment step	LAB (M17)	LAB (MRS)	Yeasts (YGC)	Moulds (YGC)	NLAB (PCA)
A	start	4.6±0.2	4.1±0.1	<1	3.3±0.1	4.5±0.1
	2	9.3±0.1	9.4±0.2	3.0±0.1	3.2±0.2	9.0±0.9
	4	9.4±0.7	9.5±0.2	7.8±0.5	-	9.4±0.5
	5	9.5±0.2	9.5±0.2	7.7±0.5	-	9.5±0.1
	7	9.5±0.2	9.4±0.7	4.1±0.8	-	8.4±0.1
B	start	4.8±0.0	4.9±0.3	3.7±0.2	-	6.6±0.8
	2	9.7±0.9	9.7±0.1	4.6±0.1	-	6.6±0.8
	4	8.8±0.2	9.2±0.1	6.2±0.8	-	5.1±0.1
	5	10.5±0.6	11.6±0.8	5.7±0.2	-	5.8±0.5
	7	11.9±0.8	12.6±0.8	8.1±0.1	-	6.3±0.1
C	start	3.7±0.5	3.8±0.1	<1	-	3.8±0.2
	2	9.4±0.1	9.3±0.4	<1	3.8±0.2	8.9±0.8
	4	8.2±0.1	9.1±0.3	6.3±0.6	3.8±0.5	9.0±0.3
	5	8.8±0.1	8.7±0.5	6.9±0.5	-	8.9±0.5
	7	9.3±0.8	9.4±0.1	5.4±0.8	-	9.3±0.7
D	start	4.9±0.1	4.1±0.5	<1	2.7±0.0	5.1±0.5
	2	8.9±0.4	8.2±0.4	<1	3.1±0.6	7.7±1.1
	4	9.7±0.8	8.9±0.8	5.5±0.1	-	9.1±0.4
	5	9.5±0.2	9.6±0.1	7.1±0.1	-	9.0±0.2
	7	10.1±0.8	9.6±0.1	7.2±0.8	-	8.3±0.2
E	start	4.5±0.1	3.9±0.1	<1		4.7±0.1
	2	9.7±0.5	8.9±0.8	2.7±0.0	3.5±0.5	7.2±0.8
	4	9.9±0.1	9.2±0.5	6.8±0.1	4.1±0.3	8.1±0.8
	5	10.1±0.6	9.3±0.2	7.5±0.2	-	8.5±0.5
	7	10.5±0.8	9.3±0.1	7.7±0.7	-	8.5±0.3

For the identification of yeast strains, the Internal Transcribed Spacer (ITS) was amplified, and all isolates were grouped in 4 clusters, characterized by a PCR product of 380, 430, 630 and 850 base pairs respectively. An ITS of 850 bp is typical of the genus *Saccharomyces* (Valente et al, 1996), and a species-specific PCR confirmed the belonging of 69 strains to *S. cerevisiae* species. For the remaining clusters the 26S rRNA gene was amplified and sequenced. The results obtained allowed to identify the 35 strains of the first cluster as *Clavispora lusitaniae*, the 11 strains belonging to the second one as *Pichia fermentans*, and the third cluster was comprised of 59 strains of *Wickerhamomyces anomalus*.

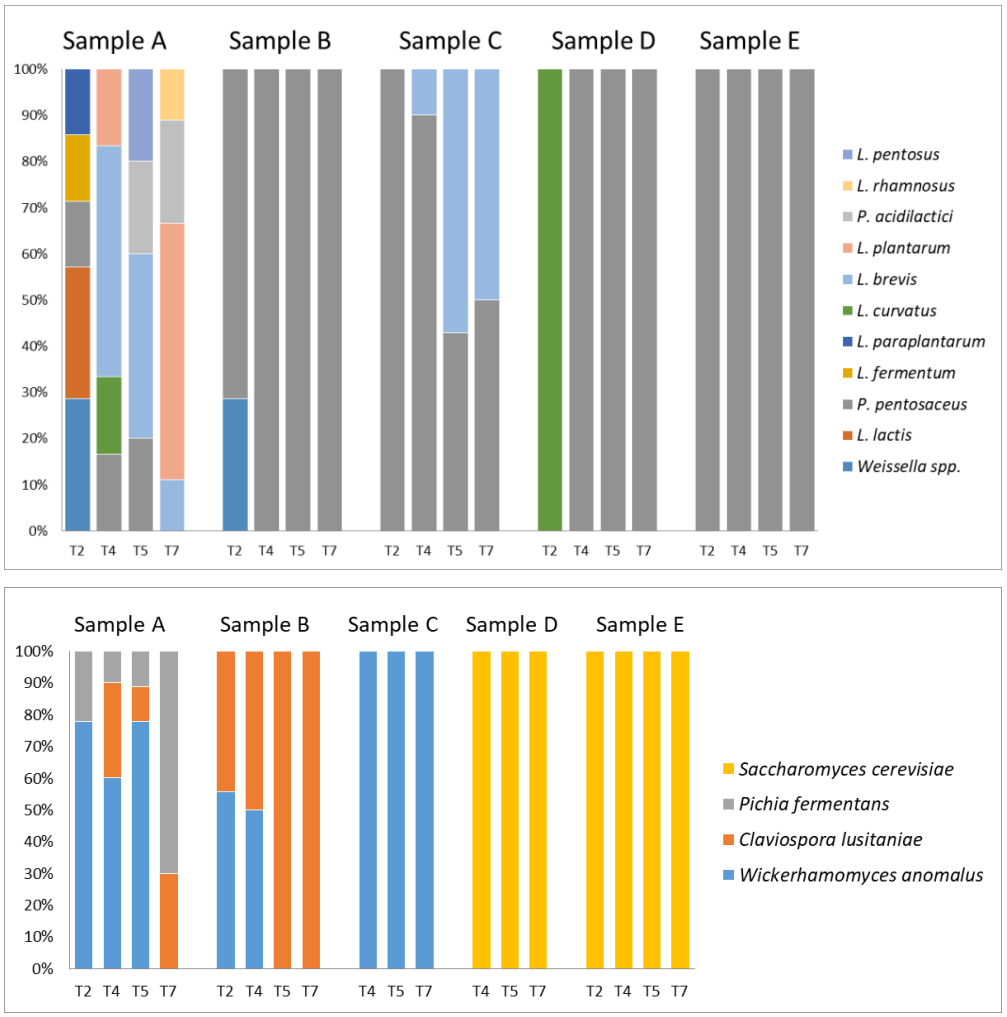
3.3.5.3 Microbial population dynamics

Microbial populations of the 5 sourdoughs are reported in Figure 1. The distribution of LAB species isolated from the 5 spelt flour samples shows the dominance of *Ped. pentosaceus* during the fermentation, representing 73% of the total LAB isolated. Particularly, strains of this species were the only isolated during all refreshment steps in sample E and after four days of back-slopping in sample B. In sample D, although initially the refreshment step was characterized by the presence of *Lact. curvatus,* from the second refreshment step, *Ped. pentosaceus* became dominant. In sample C, the final stage of the fermentation was characterized by a codominance of *Ped. pentosaceus* and *Lact. brevis.* The sample A presented a different succession of bacterial species; the composition resulted more complex, and rods dominated the fermentation. Although *Ped. pentosaceus* and *Lact. brevis* played a significant role, other species such as *Leuconostoc* spp., *Weissella* spp., *Lact. fermentum* initially, and *Lact. plantarum* and *Ped. acidilactici* in the final phases dominated the microbial community. These species have been previously reported in sourdoughs made from a range of cereals, including spelt (Van der Meulen *et al.* 2007; Coda *et al*. 2010; Manini *et al.* 2014; Decimo *et al.* 2017). However, our findings highlight the dominance of *Ped. pentosaceus* at various steeping times, suggesting that this LAB species is

the most important influential bacterial species involved in the spelt fermentation and may therefore be exploited as potential starter/adjunct culture.

Yeast communities of the 5 samples resulted in four dominant species, namely *S. cerevisiae*, *W. anomalus*, *P. fermentans* and *C. lusitaniae*. While the first three species are frequently detected in sourdough fermentation of different cereals (Carbonetto *et al.* 2018), *C. lusitaniae* is less frequently isolated from sourdoughs and considered an emerging pathogen (Gargeya *et al.*1990; Zhang *et al.* 2010). The presence of this yeast as a dominating species in sample A and especially in sample B could thus be correlated to a low quality of the flours and their appropriateness for consumption should be investigated. While samples C, D and E led to the isolation of only one yeast species, *W. anomalus* in sample C and *S. cerevisiae* in samples D and E, sample A was comprised of three species, among which, *P. fermentans* that replaced *W. anomalus* in the last stage of fermentation. *W. anomal*us has been frequently isolated from spontaneous laboratory fermentations and its presence can be related to the flour natural microbiota; *S. cerevisiae* has been mainly isolated from bakery sourdoughs as opposed to laboratory spontaneous fermentations; this fact could imply a contamination from the bakery's environment, so *S. cerevisiae* could not be considered a natural component of the flour (Vrancken *et al.* 2010). This could be the case of samples D and E, where the only presence of *S. cerevisiae* could be due to a contamination of the flour in the producing facility. *P. fermentans* is not reported as a dominant species in traditional sourdoughs, but it seems play an important role in wheat bran fermentation (Manini *et al.* 2014) and in Boza, a Turkish cereal-based beverage (Caputo *et al.* 2012).

Figure 1: Dynamics of the main LAB and yeast genera and species occurring during sourdough-like fermentation of the five spelt flours.



3.3.5.4 Sourdough fermentation-related characterization

A subset of four strains of *S. cerevisiae*, *P. fermentans*, *W. anomalus*, in comparison with a commercial baker's *S. cerevisiae* strain, and eight *P. pentosaceus* strains, were studied for some characteristics related to sourdough fermentation. Regarding yeasts (Table 3), *P. fermentans* seems the species with less adaptability to different carbon sources, being unable to ferment sucrose and maltose. However, the lack of maltose fermentation

ability could involve a non-competitive interaction with maltose-positive LAB. *W. anomalus* shows the better osmoadaptation, being able to grow in presence of 30% of the four carbon sources tested. Moreover, together with *S. cerevisiae* strains, *W. anomalus* shows a high tolerance to low pH values. The presence of acetic acid did not limit the growth of *P. fermentans*, and at a minor extent, that of *S. cerevisiae* strains.

Table 3 Carbon source utilization, osmotolerance, growth at low pH (2.5 and 3.5) and growth in presence of 1% acetic acid of selected yeast strains.

Species	Strain	Carbon source (1%)				Osmotolerance (30%)				pH		acetic acid
		Glu	Fru	Sucr	Malt	Glu	Fru	Sucr	Malt	2.5	3.5	
W. anomalus	FL 11	+	+	+	+	+	+	+	+	+	+	-
	FL 12	+	+	+	+	+	+	+	+	+	+	-
	FL 15	+	+	+	+	+	+	+	+	+	+	-
	FL 34	+	+	+	+	+	+	+	+	+	+	-
P. fermentans	CRL 42	+	+	-	-	+	+/-	-	-	-	+	+
	CRL 49	+	+	-	-	+	+/-	-	-	-	+	+
	CRL 22	+	+	-	-	+	+/-	-	-	-	+	+
	FL 10	+	+	-	-	+	+/-	-	-	-	+	+
S. cerevisiae	D8	+	+	+	+	-/+	-	+/-	-/+	+	+	+
	F1L10	+	+	+	+	-/+	-	+	+/-	+	+	-/+
	B8	+	+/-	+	+	+	+/-	+	+/-	+	+	+
	F2L20	+	+	+	+	-/+	-	+	-	+	+	-
	BS*	+	+	+	+	-/+	-	+	+/-	-/+	+	-/+

* BS strain: commercial baker's yeast

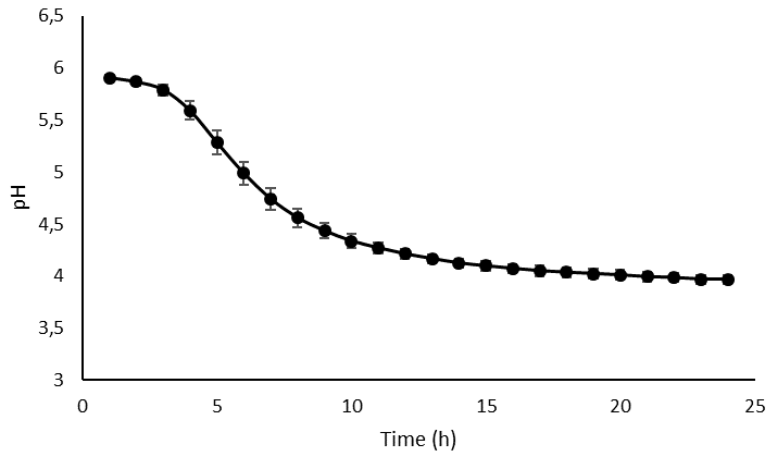
Regarding *Ped. pentosaceus*, all tested strains were able to ferment glucose, fructose and maltose (Table 4). Sucrose utilization resulted to be a strain-specific characteristic. This aspect is well-known for this species, as sucrose-utilization plasmids have been widely described for the genus *Pediococcus* (Naumoff and Livshits 2001). All strains showed a good osmoadaptation to high carbon source concentrations and a good acidification ability: the rate of acidification was high during the initial 10 h of incubation (ΔpH 1.5), and then stabilizes at a pH value of 4.0 within the 24 h of incubation (Fig. 2). The high

acidification activity after the first hours of fermentation is desired as it acts on bread structure. On the contrary, the ability to grow at low pH values is poor. This implies that the growth of the strains decreases and/or ceases when dough reaches a pH value < 4. Another characteristic that could be exploited is the autolysis ability, with the release of a pool of enzymatic activities of interest. The *Ped. pentosaceus* strains tested show a good degree of autolysis at pH 6.5, that decreases with the lowering of the pH.

Table 4 Carbon source utilization, osmotolerance, growth at low pH (2.5 and 3.5). growth in presence of 1% acetic acid and autolytic ability of the *Pediococcus pentosaceus* strains tested.

Strain	Sugar (1%)				Sugar (30%)				pH		acetic acid	Autolysis (%)	
	Glu	Fru	Sucr	Malt	Glu	Fru	Sucr	Malt	2.5	3.5		pH 4	pH 6.5
PPM1	+	+	+	+	+	+	+	+	-	-	+	15.5	34.8
SSE136	+	+	+	+	+	+	+	+	-	-	+	0.0	36.2
CR35	+	+	-	+	+	+	-	+	-	-	+	1.9	28.6
CM73	+	+	-	+	+	+	-	+	-	+/-	+	14.8	29.8
FB14	+	+	+	+	+	+	+	+	-	-	+/-	16.4	34.9
FB22	+	+	+	+	+	+	+	+	-	+/-	+	2.9	27.1
MB31	+	+	-	+	+	+	-	+	-	-	+/-	10.0	36.9
MB33	+	+	-	+	+	+	-	+	-	-	+	17.0	38.2

Fig. 2 Changes in pH during growth of the *Pediococcus pentosaceus* isolates tested in MRS broth.



In conclusion, this study is, to our knowledge, the first report that highlights the biodiversity and dynamics of microbial communities involved in sourdough fermentation of spelt, with specific regards to yeast population. Four dominant yeast species were recovered, including *C. lusitaniae*, whose role and safety deserve further studies. Compared to commercial baker's yeast, autochthonous *W. anomalus*, *P. fermentans* and *S. cerevisiae* strains show a good performance, and their use could be an advantage for their acquired adaptation to the environment, providing the stability of the fermentation process. Moreover, although documented before as a component (Van der Meulen *et al.* 2007; Weckx *et al.*2010), the paper is, to our knowledge, the first report of a spelt sourdough fermentation dominated by *Ped. pentosaceus*, a homofermentative LAB species, characterized by a high acidification rate, ability to utilize a wide range of carbon sources and to grow in high osmolarity condition. Thus, the data obtained provides the basis for the selection of autochthonous mixed cultures, with the aim of improving the nutritional potential of spelt and its rheological and breadmaking properties. This will allow the incorporation of spelt flour in bakery products formulations, with wheat flour or as a substitute to wheat flour.

Conflict of interest

The authors declare no conflict of interest

3.3.6 References

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3.4 A polyphasic approach to characterize *Weissella cibaria* and *Weissella confusa* strains

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Running headline: *Weissella cibaria* and *W. confusa* characterization

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3.4.1 Abstract

Aim: To study *Weissella cibaria* and *W. confusa* strains, naturally present in food products, but not yet included in Qualified Presumption of Safety list of European Food Safety Authority (EFSA).

Methods and Results: We carried out a comparative genome analysis of 23 sequenced *W. cibaria* and seven *W. confusa* genomes, in parallel with a physiological and functional characterization of several strains previously isolated from sourdough-like maize bran fermentation. The genome analysis revealed absence of dedicated pathogenicity factors. Some putative virulence genes found in *Weissella* genomes were also present in other Lactic Acid Bacteria (LAB) strains, considered safe by the European Food Safety Authority (EFSA) and commonly used as probiotics. The physiological tests carried out on our strains corroborated the genomic results. Moreover, interesting functional and pro-technological traits were identified: the majority of tested strains displayed high acidification rate, high reducing ability, production of exopolysaccharides (EPS), arabinoxylan degradation ability, growth in presence of fructo-oligosaccharides (FOS), bile and gastric juice tolerance and antifungal activity.

Conclusions: These results provide evidence for the possible use of selected strains of *W. cibaria* and *W. confusa* in food sector.

Significance and Impact of the Study: This polyphasic study brings new knowledge on the functional and pro-technological characteristics of these controversial species of LAB. This knowledge is an important tool for designing new selected cultures included in the Qualified Presumption of Safety (QPS) list required for food applications.

Keywords: *Weissella cibaria*, *Weissella confusa*, comparative genomic analysis, virulence traits, functional characteristics, antifungal activity.

3.4.2 Introduction

The different species ascribed to the genus *Weissella* are known for a long time for their presence in various food matrices and in many spontaneous fermentation processes of vegetables and cereals, in particular the species *W. cibaria* and *W. confusa* (Fusco *et al.* 2015; Decimo *et al.* 2017; Fessard and Remize 2017). Their frequent presence indicates that they are part of the natural microbial population characterizing different fermented products. Nevertheless, *W. cibaria* and *W. confusa* are not yet used as selected starter cultures, are not included in the list of taxonomic units proposed by EFSA for QPS status, and no specific antibiotic breakpoints for these species were suggested by the Clinical and Laboratory Standards Institute (CLSI) or the EFSA.

The scientific literature on *Weissella* spp mainly refers to the production and characterization of EPS (Di Cagno *et al.* 2006; Katina *et al.* 2009; Ahmed *et al.* 2012; Wolter *et al.* 2014; Hu and Ganzle 2018). The use of EPS synthesized by starter cultures is a common practice in the dairy industry and, in sourdough fermentation, improves the texture and storage life of bread. Moreover, *W. cibaria* and *W. confusa* are able of producing *in situ* high molecular weight dextrans. These homopolysaccharides improve the softness of fresh bread, and their use is promising in gluten-free baking (Wolter *et al.* 2014).

Other studies on *Weissella* spp. are fragmentary. However, from these publications it is clear that, although strain-specific, other properties are of interest, both for the quality and safety of food, such as the production of bacteriocins (Srionnual *et al.* 2007; Masuda *et al.* 2011), the ability to overcome the gastric barrier (Le and Yang, 2018) and to inhibit micotoxinogenic moulds (Valerio *et al.* 2009; Ndagano e*t al.* 2011). In this regard, a strain of *W. cibaria* has been used in probiotic yoghurt to reduce aflatoxin poisoning among Kenyan children (Nduti *et al.* 2016). These characteristics suggest a possible use of specific strains as potential probiotic

cultures, also supported by the hypothesis that the genus may represent a common inhabitant of our intestine (Lee *et al.* 2012).

On the other hand, *Weissella* species have been associated to possible human infections (Kamboj *et al.* 2015), even though their clinical significance remains unclear, as they have been mainly associated to polymicrobial infections and/or to immunocompromised patients. Little information is available on the mechanism and factors related to their pathogenicity, apart from the intrinsic resistance to vancomycin. The genus has also been related to diseases in animals, mainly in fish, in which its ability to cause disease has been demonstrated (Figueiredo *et al.* 2012).

Today, highlighting possible virulence factors is easier, for the availability of genomes sequenced and deposited in public databases. However, genomic data on *Weissella* are restricted to a few publications (Abriouel *et al.* 2015; Figueiredo *et al.* 2015; Li *et al.* 2017). These comparative genome analyses highlighted several genes putatively involved in virulence, such as genes encoding haemolysins, collagen adhesins and antibiotic resistance-encoding genes. The role of these genes and their transferability in *Weissella* is still unknown. In fact, the presence of some adhesins, considered a virulence factor in pathogenic microorganisms, may be a desirable feature in probiotic bacteria: a fibronectin-binding protein (FbpA) in *W. cibaria* inhibits biofilm formation of *Staphylococcus aureus* (Wang *et al.* 2017), while mucus-binding proteins may play an important role in the adhesion of the probiotic strains to the host surfaces. Comparative genomic studies on *W. cibaria* focused the attention on useful metabolic traits, such as the bacteriocin gene cluster, dextransucrase genes and genes related to an efficient proteolytic system. No specific virulence factor genes were detected (Lynch *et al.* 2015).

It follows that an in-depth study of the physiological and genetic characteristics of the species of *Weissella* could help to select suitable strains for which to assess the status of QPS and possible practical applications.

In a previous work based on the characterization of the native population of natural fermentation of maize bran (Decimo *et al.* 2017), different strains of *W.*

cibaria and *W. confusa* were isolated. They were found mainly in the last refreshment steps, where their presence was dominant. The aim of the present work was a deepened study of these isolates, with particular regard to functional and pro-technological properties. In parallel, a comparative genome analysis of 23 sequenced *W. cibaria* and seven *W. confusa* genomes available to date was carried out.

3.4.3 Materials and methods

3.4.3.1 Bacterial strains and growth conditions

Twelve strains of *Weissella cibaria* and eight strains of *W. confusa* previously isolated from sourdough-like maize bran fermentation were used in this study (Decimo *et al.* 2017). The strains were routinely sub-cultured in MRS broth/agar (Difco Lab., Augsburg, Germany) medium for 24-48 h at 30 °C. The strains were deposited in the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy, at −80 °C in MRS with 15% glycerol. Growth in milk was studied using 9% RSM (Reconstituted Skim Milk- Difco) incubated at 30 °C.

3.4.3.2 Growth at different cultural conditions

The growth performance of the strains was evaluated in MRS broth at 10 and 45 °C, at pH 9.6 and with the addition of 4.0 and 6.5% NaCl. Growth was evaluated by measuring the increase in absorbance at 600 nm (A_{600}).

3.4.3.3 Acidifying activity

Each strain was inoculated at 1% in MRS broth and in RSM. The pH was measured and recorded automatically, throughout the 24 h incubation period at 30 °C.

3.4.3.4 Redox potential

The variation in redox potential was determined according to Brasca *et al.* (2007). Strains were inoculated in MRS and incubated at 30 °C under static conditions. The oxidoreduction values were recorded every 30 min for 24 h, using a redox meter (pH302 Hanna Instruments, Villafranca Padovana, PD, Italy). The redox electrodes were standardized using two redox solutions (240 mV and 470 mV; Hanna Instruments). The Eh values were calculated according to Jacob (1970). The reduction activity was evaluated by

determining the maximum difference between two measures [Dmax (mV)] over 24 h.

3.4.3.5 Carbohydrate fermentation assay

Weissella strains were tested for the ability to ferment glucose, xylose, L-arabinose, trehalose, sucrose, lactose, ribose and galactose. Bacterial cells, grown in MRS broth at 30 °C for 16 h, were harvested by centrifugation (5000 *g*, 15 min, 4 °C), washed twice with sterile saline solution (NaCl 0,85%) and resuspended in the same volume of diluent. The fermentation assay was performed in microtiter plates containing 200 µl of Basal Sugar Medium (BSM) broth (containing g l^{-1}: polypeptone 15, yeast extract 6, tween80 1 ml, chlorophenol-red 0.04, pH 6.4) and 1% washed cellular suspensions. Carbon sources were sterilized separately by filtration and added to the sterile BSM to obtain a final concentration of 5 g l^{-1}. The plates were incubated at 30 °C and visually examined for colour change after 24 and 48 h of incubation.

3.4.3.6 Arabinoxylans degradation

β-xylosidase and α-L-arabinofuranosidase activities were assayed using 5 mmol l^{-1} *p*-nitrophenyl-β-D-xylopyranoside and 5 mmol l^{-1} *p*-nitrophenol-α-L-arabinofuranoside (Megazyme Int Ireland Ltd., Bray, Ireland) as substrates, respectively, as described by Poutanen and Puls (1988), with slight modifications. Strains were inoculated in 10 ml glucose-free base MRS medium, added with 0.5% D-xylose and 0.5% L-arabinose and incubated at 30°C for 48h. After incubation, cells were harvested by centrifugation, resuspended in 500 µl of 50 mmol l^{-1} citrate phosphate buffer at pH 5.5, disrupted using a bead beater (PRECELLYS®24-DUAL lyser/homogeniser, Bertin-technologies, France.). 200 µl of the cell suspension obtained or 200 µl of the supernatant were added to 700 µl of phosphate citrate buffer at pH 5.5 and 100 µl of substrate. The reaction was carried out at 37°C. The OD_{400} was determined. One unit of activity was defined as the amount of enzyme which produces 1 µmol of *p*-nitrophenol min^{-1} mg^{-1} protein (molar extinction

coefficient of nitrophenol = 18.000 mol l^{-1} cm^{-1}). Protein content was determined by the Bradford method (Bradford, 1976) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard.

For the assay of endo-1,4-β-xylanase activity, cell cultures were inoculated in MRS broth w/o glucose, added with 1.5% xylose, or 1% xylose and 0.2% glucose, and a 60 mg tablet of azurine-crosslinked wheat arabinoxylan (Megazyme). After 48 h of incubation at 30°C, the suspension was vortexed, filtered, centrifuged at 5000 *g* for 5 min to separate bacterial cells and the optical density of the supernatant was measured at 590 nm. The absorbance increase was correlated to the release of soluble fragments, i.e. to the enzymatic activity.

3.4.3.7 FOS utilization

To assess the ability of the strains to ferment prebiotic substances, cells grown in MRS broth at 30 °C for 16 h were harvested by centrifugation (5000 *g,* 15 min, 4 °C), washed twice with saline solution and inoculated in 5 ml MRS basal medium (MRS without carbohydrates) added with fructose and FOS (Actilight, Tereos, Lille, France) to obtain a final concentration of 10 g l^{-1}. Fructose and FOS were autoclaved separately (112 °C for 30 min). After 24 h of incubation at 30 °C, growth was evaluated by measuring the increase in absorbance at 600 nm (A_{600}).

3.4.3.8 Screening for EPS production

EPS biosynthesis was evaluated as described by Bounaix *et al*. (2009) with slight modifications. Strains were streaked on agar plates containing modified MRS medium containing 60 g l^{-1} sucrose (namely MRS-sucrose) and incubated at 37 °C for 48 h. Mucoid growth exhibiting slime production was evaluated.

3.4.3.9 Hydrophobicity assay

Bacterial adhesion to hydrocarbons was determined according to Kos *et al.* (2003) with slight modifications. Bacteria grown in MRS broth at 30 °C for 24 h were harvested by centrifugation (5000 *g*, 15 min, 4 °C), washed twice in sterile saline solution and resuspended in 0.1 mol l^{-1} KNO_3 (pH 6.2) to approximately 0.5 A_{600} (A_0). 1 ml of xylene was added to 3 ml of cell suspension. After 10-min of pre-incubation at room temperature, the two-phase system was mixed by vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature, and its A_{600} (A_1) was measured. The percent adhesion was calculated using the relation of the absorbance at 600 nm measured before and after the contact with the xylene through the following formula: Adhesion (%) = $[1 – (A_1 / A_0)] \times 100$.

3.4.3.10 Bile tolerance

Bile tolerance was measured in MRS broth containing 0.3% or 1% Oxgall (Sigma–Aldrich, Steinheim, Germany), inoculated and incubated at 30 °C. The growth was evaluated by measuring the absorbance at 600nm (A_{600}). The results were expressed as % residual growth, compared to the control.

3.4.3.11 Tolerance to simulated gastric juice

The method of Charteris *et al.* (1998) was used with slight modifications. Overnight cultures (4 ml) were centrifuged (5000 *g*, 15 min, 4 °C), washed twice in 50 mmol l^{-1} K_2HPO_4 (pH 6.5) and resuspended in 4 ml of the same buffer. One milliliter of washed cell suspension was harvested by centrifugation and resuspended in 10 ml simulated gastric juice (pepsin 0.3% w/v, NaCl 0.5% w/v) adjusted to pH 2.5 and 3.0. Total viable counts were performed on MRS agar before and after an incubation period of 1 and 3 h (for pH 2.5 and 3.0, respectively) at 37 °C.

3.4.3.12 Antifungal activity

The *Weissella* strains were tested for their antifungal activity against *Fusarium verticillioides, Mucor circinelloides, M. irregularis* and *Aspergillus flavus*. The moulds (from the Collection of the Department of Health, Animal Science and Food Safety, University of Milan, Italy) were grown on Malt Extract Agar (MEA) (Merck, Darmstadt, Germany) at 25°C for 5–7days. Then, spore suspensions were harvested by adding 15 ml of sterile milli-Q water and counted by flow cytometer estimation (BD Accuri C6 Flow Cytometer, BD Biosciences, Franklin Lakes, NJ USA). Antifungal activity was evaluated with an overlay assay (Quattrini *et al.* 2018). After growth for 16 h in MRS broth at 30 °C, the *Weissella* strains were inoculated in 2-cm lines on MRS agar plates. After incubation for 48 h at 30 °C, plates were overlaid with cooled soft (0.7%) MEA containing mould spore suspension (10^4 spores ml^{-1}) and incubated for 4 days at 25 °C. The antifungal activity was evaluated as clear zones of inhibition around the bacterial smears.

3.4.3.13 Antibiotic resistance

Antibiotic susceptibility tests were performed by the microdilution method in MRS broth incubated at 30 °C for 24 h. The minimum inhibitory concentration (MIC) was calculated after growth in MRS broth at 30 °C, using 10^5 cells ml^{-1} as initial inoculum. Interpretative criteria for susceptibility status were the CLSI guidelines and the microbiological breakpoints defined by EFSA (FEEDAP, 2012). Since a breakpoint has not been indicated for the genus *Weissella*, we considered the values reported for *Lactobacillus* and *Leuconostoc* together, as the most phylogenetically related to *Weissella* genus (Collins *et al.* 1993).

3.4.3.14 Biogenic amine production

The ability of biogenic amine production by *Weissella* strains was performed carrying out a screening plate method as reported by Bover-Cid and Holzapfel (1999). The enzymatic decarboxylation of histidine, lysine, ornithine and tyrosine was investigated after 24 h at 37 °C. *Morganella morganii* DSMZ

30164^T was used as positive control. Positive reactions for decarboxylase activity of strains were recorded when a purple colour halo occurred in response to a pH shift of the bromocresol purple indicator.

3.4.3.15 Data source for comparative genome analysis

Information of available *W. cibaria* and *W. confusa* genomes was retrieved from the National Center for Biotechnology Information (NCBI, available at http://www.ncbi.nlm.nih.gov/). Genome analysis was carried out using the Rapid Annotation using Subsystem Technology (RAST) Server (Aziz *et al.* 2008). The NCBI BLAST software was used for sequence similarity search (Altschul *et al.* 1997).

3.4.3.16 Statistical analysis

Three independent replicates of all experiments were done, and data are reported as mean values ± standard deviation.

3.4.4 Results

3.4.4.1 Comparative genomic analysis

A comparative genomic analysis on 23 sequenced *W. cibaria* and seven *W. confusa* genomes was carried out. The search of hypothetical virulence factors showed the presence of genes that could be involved in pathogenicity. In particular, *W. cibaria* and W. *confusa* genomes harboured three genes encoding putative haemolysin-like proteins, *hlyIII, hlyC* and *tlyA*. The *hly III* gene product (WP_043707670.1) is described as a predicted membrane channel-forming protein YqFA, of the haemolysin III family. The related gene was present in all genomes tested and its nucleotide sequence seemed highly conserved among the genomes (99 %). The multiple alignments indicated that YqFA, largely distributed among Gram positive bacteria, showed similarity with haemolysin III of several genera as *Leuconostoc* (WP_004911898.1) (64%), *Lactobacillus* (WP_107739861.1) (45%), *Enterococcus* (OJG68906.1) (43%), *Listeria* (WP_036096723.1) (41%). The haemolysin C gene is annotated as *hlyC*/*CorC;* analysing the conserved domain, the protein (WP_043711320.1) is classified as HlyC/CorC family transporter similar to magnesium and cobalt efflux protein CorC. The *tlyA* gene is annotated as a RNA methyltransferase (WP_043707612.1). In some pathogenic microorganisms, this protein is also supposed to show haemolytic activity (Rahman *et al*. 2010). However, no identity was found between tlyA of *Weissella confusa/cibaria* and tlyA of pathogenic microorganisms, whereas 67-74% identity was found with tlyA of several *Leuconostoc* (WP_014325058.1) and *Lactobacillus* species (WP_111443130.1).

Among the candidate genes encoding cell surface adhesins, we found one annotated gene encoding a putative fibronectin binding protein (WP_010373731.1), detected in all *W. cibaria* and *W*. *confusa* genomes, with amino acid identity of the protein very high (94%). The alignments resulted in high identities in close related genera as *Leuconostoc* (WP_036068220.1)

(60%), *Lactobacillus* (WP_017261841.1) (56%), *Pediococcus* (WP_057748137.1) (54%).

Regarding antibiotic resistance determinants, *Weissella* spp., like many lactic acid bacteria, possess an intrinsic resistance to vancomycin. This antibiotic interferes with precursors of peptidoglycan synthesis, binding D-Ala/D-Ala dipeptide, inhibiting the polymerization. In *Weissella*, the terminal D-Ala is substituted with a D-lactate or D-Ser, avoiding the antibiotic to bind to that site, and conferring the resistance phenotype (Gueimonde et al. 2013).

A multidrug efflux pump related to fosfomycin resistance was found in all genomes analysed (CP012873.1). Further analysis showed that the efflux pump is widespread in different Gram-positive and Gram-negative genera, *Leuconostoc* (WP_036067854.1) (66% amino acid identity), *Lactobacillus* (WP_010622689.1) (58%), *Listeria* (WP_096926801.1) (50%), *Staphylococcus* (WP_000610059.1) (42%), *Salmonella* (WP_050189798.1) (34%) *Escherichia* (WP_001612799.1) (29%). Taking into account other published data regarding both *Weissella* and other Gram positive and negative bacteria, fosfomycin resistance could be considered intrinsic (Arca et al. 1997; Ayeni et al. 2011; Abriouel et al. 2015).

No genes related to tetracycline resistance were found in the genomes of W. cibaria and *W. confusa*. In *W. cibaria* genomes one gene present in two copies was annotated as a methicillin resistance protein (OSP89428.1). Two genes exhibiting high level of similarity were also found in the *W. confusa* genomes. This protein is present in all-related Gram-positive genera (*Leuconostoc*, *Lactobacillus*, *Pediococcus*) with high identities (>80%).

No genes encoding decarboxylases, related to biogenic amines production were detected in any of the genomes analysed.

Regarding the search of functional traits, we found genes related to dextran production, well documented in *Weissella* species, and genes related to arabinoxylan catabolism, a trait not yet investigated. Dextran production is mainly correlated to dextransucrase activity (Galle et al. 2010). In the genomes analysed, dextransucrase gene was found in all *W. cibaria*

(GU237484.3) and *W. confusa* (KP729387.1) strains, with an amino acid identity of 75%. The arabinoxylan degradation is related to the activity of several enzymes, such as endo-1,4-β-xylanases, α-l-arabinofuranosidase, β-xylosidase, α-glucuronidase and feruloyl esterase. Their combined action allows the obtainment of oligosaccharides with prebiotic properties, an increase of soluble fiber and, with the action of feruloyl esterase, the increase of free ferulic acid. Twenty *W. cibaria* genomes analysed harbour the gene encoding a β-xylosidase (WP_010373933.1). This gene is also present in all *W. confusa* genomes tested (nucleotide similarity 95%). The protein exhibited significant similarity (77-84%) to the known β-xylosidases of *Leuconostoc* spp. (WP_029509980.1), *Lactococcus lactis* (WP_043735157.1) and *Lactobacillus oligofermentans* (WP_057890071.1). The gene encoding the feruloyl esterase was not detected. The other genes related to arabinoxylans degradation were differently distributed. Only *W. cibaria* genomes harboured a gene encoding an α-N-arabinofuranosidase (WP_128736204.1). The alignment resulted in high amino acidic identity with α-N-arabinofuranosidase of *Weissella bombi* (WP_092461590.1) (80%), *Lactococcus lactis* (WP_058219862.1) (76%), *Lactobacillus brevis* (WP_021741280.1) (75%), *Pediococcus acidilactici* (WP_063504605.1) (75%). On the other hand, the endo-1,4-β-xylanase gene was only present in *W. confusa* genomes. *W. confusa* β-xylanase (WP_112464318.1) exhibited similarity to xylanases of *Bifidobacterium* spp. (WP_118292990.1; 47-48%identity) and *Clostridium* spp. (WP_077837723.1; 47-49%). Therefore, it is possible to hypothesize that the degradation of arabinoxylans could be obtained by a potential synergistic action of selected strains of the two species, which are isolated most of the times from the same fermented cereal products (Bjorkroth et al. 2002).

3.4.4.2 Characterization of *W. cibaria* and *W. confusa* strains isolated from sourdough-like maize bran fermentation

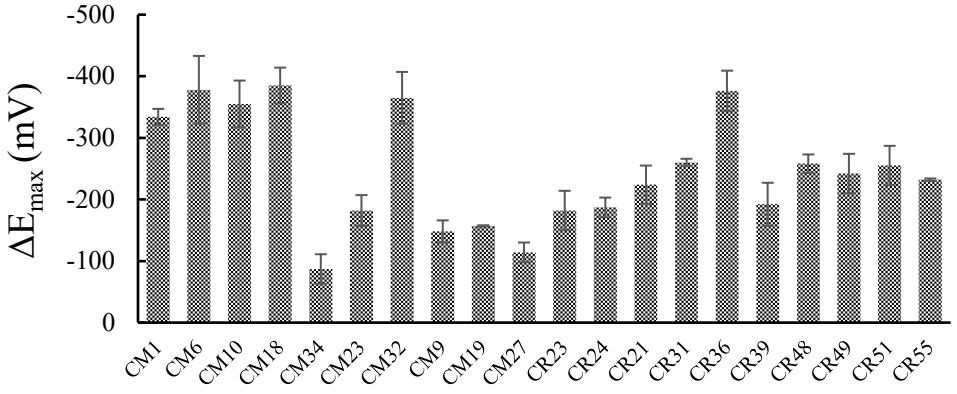
The strains tested showed a good adaptability towards cultural stresses, such as temperature, NaCl concentration and pH value. All strains were able to grow at 10°C, in presence of 4% NaCl and a pH 9.6. The growth at 45°C was more evident for *W. confusa* strains.

All strains were able to utilize glucose, xylose and sucrose as sole carbon sources. Lactose and trehalose were not fermented. Galactose and ribose were only fermented by strains belonging to *W. confusa*. This ability, which occurred after a longer incubation period (48-72 h), could be due to a selection inside the population and, for galactose, to the activation of an inducible Leloir pathway (Frey, 1996). The fermentation of L-arabinose also allowed to discriminate between the two species: all *W. cibaria* strains were able to utilize this carbon source, contrarily to *W. confusa* strains. This is in accordance with the exclusive presence of the gene-cluster for using L-arabinose in *W. cibaria* genomes. This cluster contains three genes encoding enzymes for L-arabinose catabolism: the ribulokinase, the L-arabinose isomerase, the L-ribulose-5-phosphate 4-epimerase. These enzymes catalyze the conversion of L-arabinose into D-xylulose-5-phosphate, an intermediate of the pentose phosphate pathway.

All tested strains showed a high acidification rate in MRS medium, with a pH value, after 24 h of incubation at 30°C, ranging from 3.5 to 4.9. On the contrary, a limited capacity of growing and acidifying in milk was observed, according to the inability to ferment lactose.

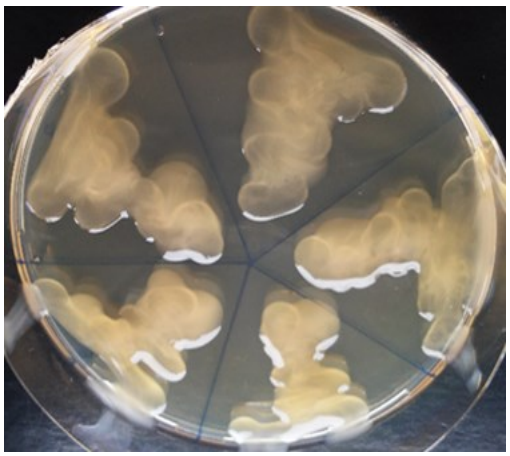
The 20 *Weissella* strains were also screened to evaluate the changing in the redox potential during the growth in MRS liquid medium. All strains had high reducing ability (Fig.1), in particular the strains CM18, CM10 (*W. cibaria*) and CR36 (*W. confusa*), which could reach values close to -400 mV.

Fig. 1 Maximum redox potential difference (ΔEmax mV) after growth of *Weissella cibaria* and *W. confusa* strains in MRS medium for 24 h at 30°C.



The ability to synthesize EPS by *W. confusa* and *W. cibaria* is well documented by many authors. In this work we qualitatively screened the ability of EPS production in solid medium added with high percentage of sucrose. All strains were able to produce EPS, resulting in sticky and viscous colonies (Fig. 2).

Fig. 2 Sticky and viscous colonies of *Weissella cibaria* CM23 in MRS-sucrose agar, after 24 h at 30°C.



Regarding arabinoxylans degradation ability, a trait not yet investigated, we found interesting results (Table 1). ß-xylosidase appeared to be present in both species, albeit the measured activity greatly varied among the strains, ranging from 0.10 to 30.22 mU. This activity was principally found in the cellular extract, indicating a presumable cell-associated activity located on the cell surface. Four *W. cibaria* strains, and three *W. confusa* strains also showed to possess α-L-arabinofuranosidase activity. The relatively poor activity could suggest a double activity of the ß-xylosidase, with a higher affinity for xylose. This aspect needs further investigation.

Endo-1,4-β-xylanase activity was not found in *W. cibaria* strains, in accordance with genome analysis; within *W. confusa*, five strains showed a moderate xylanase activity (OD increase about 0.11±0.01 units) when compared to the negative control (data not shown). This activity was recorded in presence of xylose only. When glucose was added to the medium, although bacterial growth increased, the enzymatic activity observed was lower. This fact reflects literature reports indicating xylose as a suitable inducer of the enzyme production (Kulkarni, *et al.* 1999).

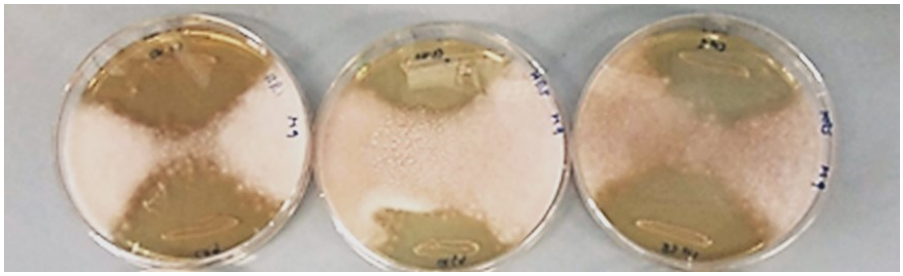
Table 1 ß-xylosidase and α-arabinofuranosidase activity of the *Weissella* strains detected in cell extracts.

Strains		**ß-xylosidase (mU)***	**α-arabinofuranosidase (mU)**
W. cibaria	CM1	0.65	-
	CM6	1.43	-
	CM10	18,32	0,58
	CM18	7,23	-
	CM34	18,77	0,39
	CM23	1,20	-
	CM32	17,25	0,03
	CM9	22,24	1,26
	CM19	25,13	0,91
	CM27	8,69	-
	CR23	0.10	-
	CR24	0.97	-
W. confusa	CR21	-	-
	CR31	8.82	-
	CR36	5.11	-
	CR39	14.78	0.45
	CR48	30.22	0.46
	CR49	11.26	-
	CR51	20.20	0.23
	CR55	16.96	-

*U= the amount of enzyme releasing 1 μmol of *p*-nitrophenol min^{-1} mg^{-1} of protein

Regarding antifungal activity all *Weissella* strains tested were able to inhibit the growth of *Fusarium verticillioides* (Fig. 3). Moreover, most of the strains analysed (17/20) exerted a strong inhibition against the aflatoxigenic *Aspergillus flavus*. Approximately 55 and 45% of the strains inhibited *Mucor irregularis* and *M. circinelloides* respectively (Table 2).

Fig. 3 Example of antifungal activity against *Fusarium verticillioides*: A, *Weissella cibaria* CM1, B, *W. cibaria* CM18, C, *W. confusa* CR55.



Regarding functional traits, we studied the ability of the tested strains to grow in presence of FOS. All *W. cibaria* and *W. confusa* strains were able to ferment fructose; FOS were used as carbohydrate source by all *W. confusa* strains and by 10/12 *W. cibaria* (Fig.4). The utilization of prebiotic compounds is one of the indicators of probiotic potential of a bacterial strain (Kaplan and Hutkins, 2000). These data highlight that, generally, these species possessed a high attitude to use prebiotics as growth source, in a future probiotic perspective.
An essential trait of a probiotic microorganism is its ability to reach and survive in the large intestine, overcoming the intestinal barrier. The strains tested showed a moderate tolerance to simulated gastric juice, many strains decreasing their viability of 3 log cycles at pH 3. In simulated gastric juice at pH 2.5 a progressive reduction in viability was observed, but recovered viability after 1 h was about 10^2-10^4 CFU ml^{-1} for all strains (Fig. 5). Moreover, all strains showed a noticeable ability to resist to bile salts, reaching values between 30% and 60% of residual growth even when the highest concentration of bile salts (1%) was added to the medium (Fig. 6). At the

concentration of 0.3%, the inhibition was minimal, with residual growth ranging from 48% to 86%.

Table 2 Antifungal activity of *Weissella* strains against *Mucor irregularis*, *M. circinelloides*, *Fusarium verticillioides* and *Aspergillus flavus*. Strains were classified as no (-) or strong (+) inhibitors by using the overlay method.

Strains		Antifungal activity			
		Mucor irregularis	*Mucor circinelloides*	*Fusarium verticillioides*	*Aspergillus. flavus*
W. cibaria	CM1	+	+	+	+
	CM6	+	+	+	+
	CM10	+	+	+	+
	CM18	+	+	+	+
	CM34	-	-	+	+
	CM23	-	-	+	+
	CM32	-	+	+	+
	CM9	-	-	+	-
	CM19	-	-	+	+
	CM27	-	-	+	+
	CR23	-	-	+	-
	CR24	-	-	+	+
W. confusa	CR21	-	-	+	+
	CR31	-	-	+	+
	CR36	+	-	+	-
	CR39	+	+	+	+
	CR48	+	+	+	+
	CR49	+	+	+	+
	CR51	-	-	+	+
	CR55	+	+	+	+

Fig. 4 Growth of *Weissella cibaria* and *W. confusa* strains in presence of FOS. Growth was evaluated by measuring the increase in absorbance at 600nm (A_{600}). ■ MRS basal medium; ▒ MRS + FOS; ▨ MRS + fructose.

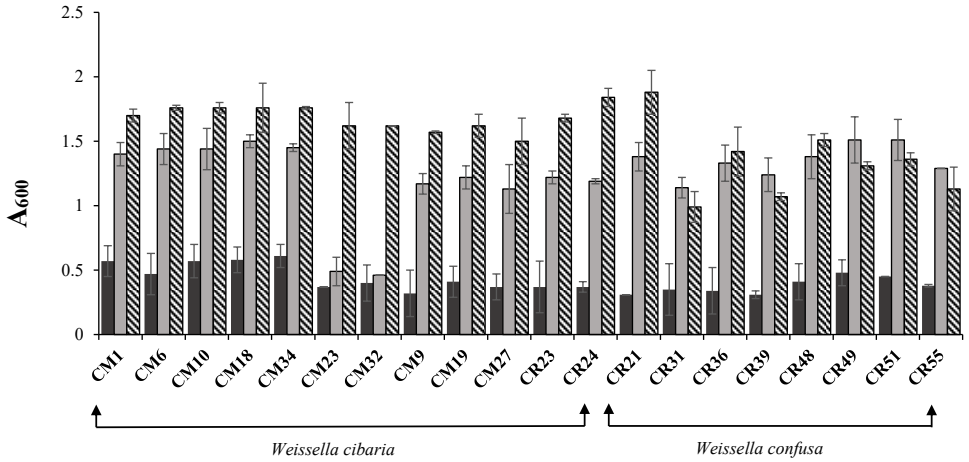
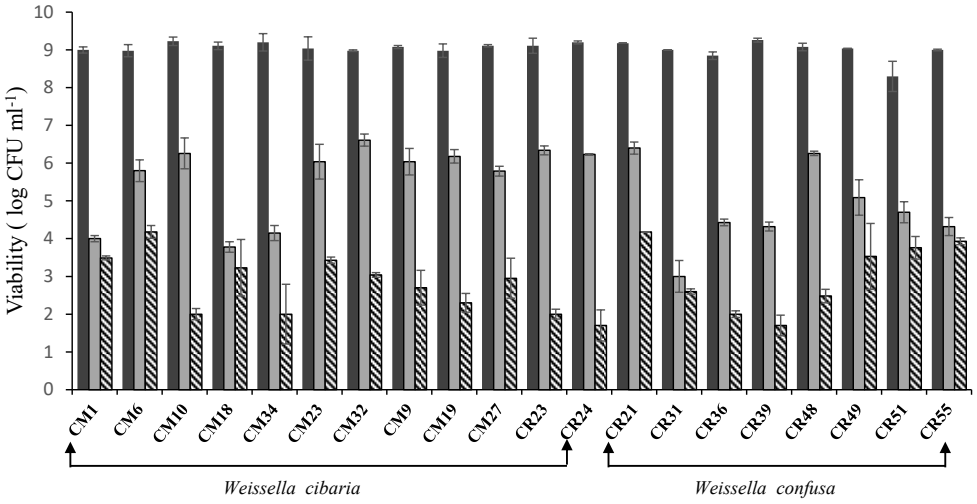


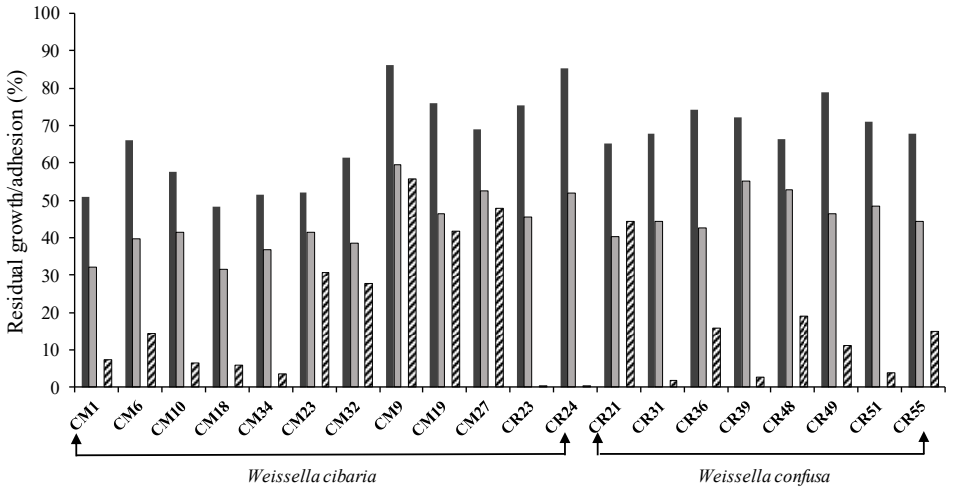
Fig. 5 Viability of *Weissella* cibaria and *W. confusa* strains under influence of simulate gastric juice. ■ control; ▒ viability after 3 h at pH 3; ▨ viability after 1 h at pH 2.5.



Hydrophobicity properties of the strains were evaluated as percentage of adhesion to a hydrophobic solvent, the xylene. This bacterial trait could be predictive of adhesiveness of probiotic bacteria, the first step of the

colonization of the epithelium. The results are shown in Fig. 6. Generally, the data obtained indicate a low potentiality of the strains to adhere to the solvent, even though this ability could be considered strain-specific: high values (55.6, 48.0 and 44.3%) were obtained for strains CM9, CM27 (*W. cibaria*) and CR21 (*W. confusa*), respectively.

Fig. 6 Tolerance to bile salts (expressed as % residual growth) in presence of 0.3% ■ and 1% ■ Oxgall, and % adhesion to xylene ▨



Antibiotic resistance is regarded with increasing attention from EFSA and OMS, for the spread of microbial resistances. To test antibiotic resistance profiles of the strains, we considered the breakpoints established by EFSA (FEEDAP, 2012) for *Leuconostoc* and *Lactobacillus*, which are the closest genera to *Weissella*. Indeed, up to now no specific antibiotic breakpoints for these species have been suggested by the CLSI or the EFSA. The strains were identified as either sensitive (MIC ≤ breakpoint) or resistant (MIC > breakpoint) according to the following breakpoints (μg ml^{-1}): tetracycline 8, streptomycin 64, kanamycin 64, gentamycin 16, lincomycin 8, chloramphenicol 4, trimethoprim-sulfamethoxazole 8+160, oxacillin 4,

ampicillin 4. Since intrinsic resistance to vancomycin and fosfomycin are known, these antibiotics were not tested. Results showed that all tested strains were susceptible to tetracycline, ampicillin and chloramphenicol. Lincomycin had effect on 12/20 strains tested. On the other hand, all the strains were resistant to aminoglycosides (AG; gentamycin, kanamycin and streptomycin) and to sulphonamides. Regarding methicillin, all *Weissella* strains tested showed MIC values ranging from 4 to 8 μg ml^{-1} (break point related to methicillin resistant bacteria 4 μg ml^{-1}).

Finally, none of the *W. cibaria* and *W. confusa* strains tested showed the ability to produce biogenic amines.

3.4.5 Discussion

The polyphasic approach carried out highlighted that selected *Weissella cibaria* and *W. confusa* strains could be considered suitable candidates to obtain the QPS status and can represent interesting adjunct cultures to be exploited in the food sector and in probiotic formulations. A first supporting evidence comes from an extended comparative genomic analysis on 30 genomes deposited in databases, focalized on the search of putative virulence factors. The search showed the presence of genes that could be involved in pathogenicity, such as haemolysin-like proteins and adhesins. However, these traits are commonly found in many bacteria and are not necessarily related to virulence. Haemolysin-like proteins are ubiquitously present in many LAB and their role in pathogenicity is unknown. For these reasons, the presence of the related genes should not be an exclusion factor. Regarding adhesins, we found a gene encoding a fibronectin binding protein, highly similar to that found in closer related LAB. In the last decade, several studies have revealed that a wide range of bacteria possess adhesin-like proteins, able to bind to fibronectin, that could play a direct role in bacterial colonization and in bacteria–host interactions (Henderson *et al.* 2010). Their presence in pathogenic strains could be considered the first step of infection; on the contrary, in strains with probiotic potential this characteristic should be regarded as a key factor for the attachment of probiotic bacterial cells to the human host. Another function of this adhesin could be related to the inhibition of the colonization of host tissues by pathogenic strains, as reported by Wang *et al*. (2017), regarding the role of a fibronectin-binding protein of *W. cibaria* isolates against the colonization by *Staphylococcus aureus*. Regarding antibiotic resistance, we chose breakpoint values that generally conform to *Leuconostoc* breakpoints indicated by EFSA and suggested for *W. confusa* strains by Sturino (2018). However, considering the taxonomic position of *Weissella* between *Leuconostoc* and *Lactobacillus* genera and the data obtained on our strains, we suggest higher breakpoint values for kanamycin (

64 μg mL^{-1} instead of 16) and ampicillin (4 instead of 2). For kanamycin a more resistant phenotype for *W. confusa* strains was also indicated by Sturino (2018). The genomic analysis combined with phenotypic experiments on our strains allowed to highlight an intrinsic resistance to many antibiotics, trait present in other LAB commonly used as probiotics, and not easily transferable to other bacteria strains. We refer not only to vancomycin and fosfomycin intrinsic resistance, but also to aminoglycosides and sulphonamide resistance. All the 20 strains tested showed resistance towards aminoglycosides (AG; gentamycin, kanamycin and streptomycin). These data were in agreement with previous findings (Katla *et al.* 2001, Hertel *et al.* 2007, Hummel *et al.* 2007), showing several LAB starters and non-starters with 70-80% of resistant phenotype. The most common mechanism of AG resistance is a chemical modification by aminoglycoside-modifying enzymes (AMEs) (Garneau-Tsodikova and Labby, 2016). However, along the tested genomes available in databases no genes encoding AG-acetyltransferases, AG-nucleotidyltransferases and AG-phosphotransferases were found. For these reasons, it is possible to hypothesize other potential, acquired mechanisms of resistance, mutations of the ribosome or enzymatic modifications of the ribosome (Garneau-Tsodikova and Labby, 2016). Moreover, in LAB the intrinsic resistance to aminoglycosides has been attributed to the absence of cytochrome-mediated electron transport, which mediates drug uptake (Charteris *et al*. 2001; Hummel *et al*. 2007) In this context, our data are in agreement with other studies indicating that AG resistance may be considered intrinsic. The sulphonamide resistance can also be due to intrinsic modification of the dihydropteroate synthetase enzyme. As for aminoglycosides, many LAB show a natural reduced sensibility towards these antimicrobials since most of them lack the complete pathway of ex-novo folic acid biosynthesis (the target of the sulphonamides) (Katla *et al.* 2001). Regarding methicillin, we evaluated the MIC values for oxacillin, according to break point related to methicillin resistant bacteria (4 μg ml^{-1}), in comparison with three *Lactobacillus* strains, commercially used as probiotic cultures,

Lactobacillus rhamnosus GG, *Lactobacillus paracasei* ATCC 5622, *Lactobacillus plantarum* ATCC 4008. All *Weissella* and *Lactobacillus* strains tested showed MIC values ranging from 4 to 8 μg ml^{-1}. These values seem to indicate a methicillin resistance. A search on NCBI-CDD indicates that the genes found in *Weissella* genomes, annotated as methicillin resistance protein, encode proteins having a catalytic domain related to penicillin-binding proteins (PBPs) involved in cell wall peptidoglycan synthesis. These proteins are present in all-related Gram-positive genera (*Leuconostoc, Lactobacillus, Pediococcus*) with high identities (>80%). Methicillin inhibits the transpeptidation domain of PBPs, interfering with the cross-linking reaction, weakening the cell wall. Methicillin resistance is well studied in *Staphylococcus aureus* and related to the presence of a gene*, mecA*, encoding an altered PBP resistant to methicillin. Mechanisms of methicillin resistance other than *mec*A seem rare (Katayama and Hiramatsu, 2000; Stapleton and Taylor, 2002). The nucleotide sequence alignment of the genes found in *Weissella* genomes with the known *mecA* gene in *S. aureus* (KC243783.1) did not show homology. For these reasons, further studies are needed to fully disclose the mechanism of this supposed resistance of *Weissella* strains, towards this antimicrobial.

From the data obtained *W. cibaria* and *W. confusa* seem associated to low virulence profiles and their presence in food could be considered not only a low health risk, but also an adjunct advantage. Indeed several pro-technological and functional traits were detected in the tested strains, for both species, such as good adaptability towards cultural stresses, high acidification rate and EPS production. This last technological trait is highly requested for sourdough fermented products, such as gluten free doughs, where hydrocolloids are crucial to improve the texture and the specific volume of the bread. We also verified the reducing capacity of the strains, a parameter not yet investigated in these species. The high reducing ability shown could be exploited in sourdough environment, coupled with the traditional pH measurement, to successfully control the baking process (Capuani *et al.*

2012). It allows to control that the fermentation process is going in the prefigured direction, ensuring the desired quality of the product and monitoring possible contaminations. Moreover, the redox potential has been described to influence the aromatic profile, as well as protein structure and texture of baked products (Kieronczyk *et al*. 2006).

Another interesting trait not yet elucidated in these species has been highlighted, related to arabinoxylans degradation ability. All strains tested showed a considerable ß-xylosidase activity; moreover, at the strain level, xylanase and α-arabinofuranosidase activities have also been detected. Recently, arabinoxylan degrading enzymes have attracted considerable attention because of their biotechnological and nutritional potential in several food processes, as clarification of fruit juices and wines (Beg *et al*. 2001) and in the cereal sector for increasing the amount of soluble dietary fibers and the content of free ferulic acid (Katina *et al.* 2012). In this context, the potential showed by the tested strains deserves to be further deepened.

Antifungal activity is an appreciated additional feature of starter and adjunct cultures, in several food sectors, such as cereal sector, where moulds are widely present and can represent an extra risk for the possible production and accumulation of mycotoxins. Several publications have highlighted the positive use of LAB strains as agents of biocontrol, due to their ability to secrete compounds such as organic acids, phenyllactic acid, cyclic dipeptides, hydroxy-fatty acids, able to inhibit mould development, limiting the mycotoxin production (Lavermicocca *et al.* 2000; Quattrini *et al.* 2018). Little information is available regarding the antifungal potential of *Weissella* strains. Interestingly, all *Weissella* strains tested were able to inhibit the growth of *Fusarium verticillioides*, the most prevalent fungus infecting the maize crops, producing a wide range of mycotoxins, including fumonisin B1 (Deepa and Sreenivasa, 2017), considered the most toxic one. Moreover, most of the strains analysed exerted a strong inhibition against the aflatoxigenic *Aspergillus flavus* and inhibited *Mucor irregularis* and *M. circinelloides. Mucor* species are frequently isolated from food matrices and *M. circinelloides* is

considered one of the causal agents of the fungal infection mucormycosis (Lee *et al.* 2014).

Finally, we studied some functional characteristic poorly investigated in these species. Specifically, we evaluated the ability of the tested strains to utilize prebiotics and their ability to reach and survive in the large intestine. The data obtained are interesting: the tested strains possessed a high attitude to use prebiotics as growth source, showed a moderate tolerance to simulated gastric juice and a noticeable ability to resist to bile salts. These characteristics could be exploited in a future probiotic perspective.

In conclusion, new data on genomic, biotechnological and functional characteristics of *W. cibaria* and *W. confusa* are reported. These data suggest that *W. cibaria* and *W. confusa* strains may be suitable alternative cultures to be exploited in the food sector and in probiotic formulations. Further studies are in progress on selected strain CM9 of *W. cibaria* and strain CR48 of *W. confusa* for the obtainment of QPS status required for food applications.

Conflicts of interest

No conflict of interest declared

3.4.6 References

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3.5 Physiological performance of *Kazachstania unispora* in sourdough environments

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3.5.1 Abstract In this work we explored the potential of several strains of *Kazachstania unispora* to be used as non- conventional yeasts in sourdough fermentation. Properties not yet deepened were evaluated, as carbon source utilization, stress tolerance and the performance in fermentation. The *K. unispora* strains are characterized by rather restricted substrate utilization: only glucose and fructose supported the growth of the strains. However, the growth in presence of fructose was higher compared to a *Saccharomyces cerevisiae* commercial strain. Moreover, the inability to ferment maltose can be considered a positive characteristic in sourdoughs, where the yeasts can form a nutritional mutualism with maltose-positive Lactic Acid Bacteria. Tolerance assays showed that *K. unispora* strains are adapted to a sourdough environment: they were able to grow in conditions of high osmolarity, high acidity and in presence of organic acids, ethanol and salt. Finally, the performance in fermentation was comparable with the *S. cerevisiae* commercial strain, but the growth was more efficient, which is an advantage in utilizing these strains in an industrial scale. Our data show that *K. unispora* strains have positive properties that should be explored further in bakery sector.

Keywords · Non-conventional yeasts, *Kazachstania unispora,* sourdough fermentation, stress tolerance

3.5.2 Introduction

In the last decade, a great attention has been paid to design new microbial starters for food sector. Particularly, the demand for products with peculiar aroma profiles and improved nutritional properties led to a renewed interest into the characterization of non-conventional microbial cultures selected from spontaneous food fermentations (Steensels and Verstrepen 2014; Aslankoohi et al. 2016). The use of the sourdough process is one of the oldest spontaneous biotechnological processes in cereal food production. In sourdough preparations the autochthonous microbiota, composed of yeasts and Lactic Acid Bacteria (LAB) confers positive features to the final product, positively influencing the technological, nutritional and organoleptic properties and implementing the shelf-life of the bread (De Vuyst et al. 2016; Martorana et al. 2018). For these reasons there is a growing interest in sourdough preparations and in investigating the potential of the autochthonous microflora found in spontaneous sourdough fermentations. However, while the study and the use of LAB species has received considerable attention, the study and the use of non-conventional yeasts for bread dough fermentation has received relatively little attention. Although these yeasts do not always have the leavening ability of bread yeast, they can strongly contribute to improve the nutritional characteristics and the flavor profile of the product. They also show a higher tolerance towards stressful conditions such as pH, osmotic and oxidative stress (De Vuyst et al. 2016).

Kazachstania unispora (formerly *Saccharomyces unisporus*) (Bhattacharya et al. 2013) is a non-conventional species of yeast, belonging to a genus "*Saccharomyces sensu lato* species", which contains more of 40 different species, isolated from several habitats (Carbonetto et al. 2018). *K. unispora* has been found in traditional dairy products, and represents a characteristic species of the autochthonous microbial population of kefir, where it seems to have an active role as probiotic (Marsh et al. 2013; Bourrie et al. 2016). The species has also been found in sourdoughs, albeit to a lesser extent. Two

groups of sourdough *Kazachstania* species have been defined (Carbonetto et al. 2018). The representative species of the first group is *K. exigua*, the most frequently cited sourdough species in the literature. Within the second group, *K. unispora* remains poorly characterized.

K. unispora is able to ferment galactose but not lactose; its frequent presence in dairy products could be due to this ability, thus not competing with lactose fermenting bacteria (Montanari et al. 1995). For the same reason, *K. unispora* could be adequate to be used as selected culture in sourdough fermentation, because of its inability to ferment maltose. Indeed, maltose negative yeasts could establish a more stable consortium with LAB in sourdoughs because of a lack of competitiveness for the carbon source (De Vuyst and Neysens 2005; Venturi et al. 2012).

Besides the fermentative role, *K. unispora* produces a number of metabolites with an important impact on the sensory profile of the product, but their role in the nutritional properties is still to be studied (Bhattacharya et al. 2013). Some studies indicate the ability of strains of *K. unispora* to accumulate high amount of palmitoleic acid (Nabi et al. 2016; Gientka et al. 2017). This characteristic may be of particular interest, since the supplementation with this mono-unsaturated fatty acid has been linked to a diminished risk to develop cardiovascular diseases (Griel et al. 2008).

K. unispora do not seem to pose human health risks, as it does not grow at 37 °C. Moreover, strains of this species isolated from kefir have been associated to low virulence profiles (Lim et al. 2019). The observed resistance to the antifungal fluconazole may be regarded as intrinsic to the species, not easily transmissible, as reported for *S. cerevisiae* strains, showing reduced susceptibility to most azole agents (Kontoyiannis and Rupp 2000; Lim et al. 2019). Furthermore, to the best of our knowledge, there are no reports of infections caused by the yeast *K. unispora*, and the species is included in the Inventory of microbial food cultures with safety demonstration in fermented food products (Bulletin of the International Dairy Federation, 2018).

This study aimed to be a further exploration of the potential of the species *K. unispora*, through the evaluation of properties not yet deepened. Specifically, we characterized several *K. unispora* strains in order to contribute to the body of knowledge, with the aim to use them as alternative baking yeasts.

3.5.3 Materials and methods

3.5.3.1 Strains and growth conditions

Kazachstania unispora strains were previously isolated from fermented maize bran (11 strains named KM 1-11) and from artisanal kefir grains (5 strains named KK12-16). The identity of the strains was confirmed using *Hinf*I restriction analysis of the amplified Internal Transcribed Spacer (ITS) region, as previously reported (Decimo et al. 2017). A commercial baker's yeast purchased from AL.NA Srl (Turin, Italy) was used for comparison.

Strains were routinely subcultured in YPD broth at pH 6.0 and grown at 28°C, either in static or shaking conditions. The composition of the medium is as follows (g L^{-1}): yeast extract 10, peptone 20, glucose 20. Pure cultures were maintained on agar YPD at 4°C for short term storage, and in YPD broth supplemented with glycerol (15% v/v) at −80°C for long term storage. The cultures are deposited in the Microbial Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy.

3.5.3.2 Growth on different carbon sources

Cell growth on YP medium supplemented with 1% (w/v) of glucose, maltose, fructose and sucrose was monitored by optical density at 600 nm (OD_{600}) using a plate reader (Biotek, Vermont, USA). The plate reader was run in discontinuous mode, with absorbance readings performed in 30 min intervals and preceded by 30 sec shaking at medium speed. Cultures were grown in independent triplicates and the resulting growth data were expressed as the mean of these replicates. Carbohydrates were dissolved in water, sterilized by filtration (0.2 µm filter size) and then added to autoclaved YP. Cells from pre-cultures grown in YPD broth were used as inoculum: they were harvested during the exponential phase of growth by centrifugation, washed twice with a saline solution (NaCl 0.9% w/v) and inoculated at 1% (v/v) (starting OD_{600} between 0.06–0.08).

3.5.3.3 Tolerance assays

Pre-cultures of the strains, obtained as reported above, were used as inoculum to test the ability of the strains to tolerate different types of stressors. The evaluation of the growth was done by OD_{600} determination after 24-48 h of incubation at 28 °C in static conditions, in comparison with the growth in standard conditions. All tests were conducted in triplicates.

Temperature and pH tolerance

All strains were investigated for their ability to grow in YPD broth at different temperatures (25°, 30°, 37° and 42°C) and at different pH of the medium (2.5, 3.0, 3.5 and 4.0).

Osmotolerance

Yeast strains were cultured in YPD broth containing 10 and 30% glucose or fructose and incubated for 48 h. We also evaluated the tolerance toward two types of stressors: a low pH (pH 3.0) and low/high osmotic stress (glucose; 10% and 30%). Further, the viability of strains after incubation in YPD broth added with 2 and 6% NaCl was investigated.

Organic acids and ethanol tolerance

The ability of the strains to grow in high organic acid concentrations was tested in YPD at pH 5, supplemented with 1% (v/v) lactic acid, 1% (v/v) acetic acid or a mixture of both organic acids (0.5% + 0.5% v/v).

The resistance to ethanol was assessed by adding to the medium 4, 6 or 12% of ethanol (v/v).

3.5.3.4 Glucose and ethanol assays

Glucose and ethanol concentrations in the supernatants of the cultures grown both in static and shaking conditions were assayed in triplicate using commercial enzymatic kits (catalog no.1 076251 035, 1 0176290 035; Hoffmann La Roche, Basel, Switzerland). Two different concentrations of glucose, 20g L^{-1} and 160 g L-1, were evaluated.

3.5.3.5 Rheofermentometer assay

The performance of the strains in fermentation of dough was measured in a rheofermentometer assay, using a Chopin F4 Rheofermentometer (Chopin Technologies, Villeneuve-la-Garenne Cedex, France) at 30°C for 8h. The height reached by the dough was recorded for the *K. unispora* strain and for the commercial *S. cerevisieae* strain as a comparison.

3.5.3.6 Molecular typing

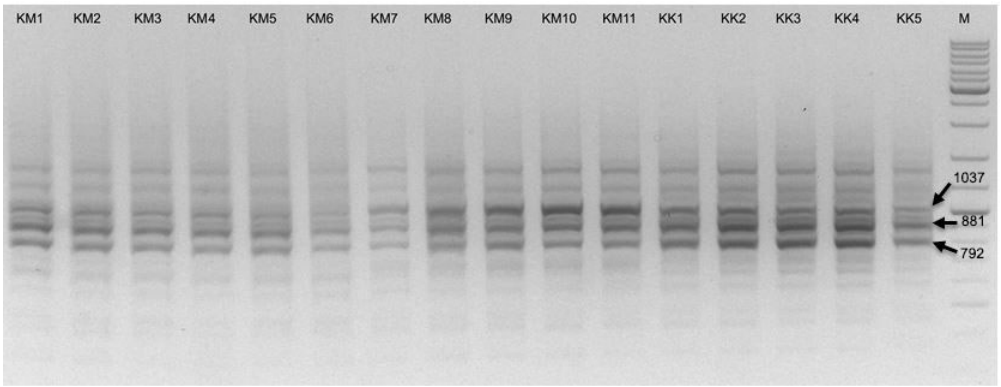
K. unispora strains were typed by random amplification of polymorphic DNA-PCR (RAPD) typing with primer (GTG)5. PCRs were performed in a 25 µL reaction mixture contained 100 ng of bacterial DNA, 2.5 µL of 10x reaction buffer (Fermentas, Vilnius, Lithuania), 200 µM of each dNTP, 2.5 mM MgCl2, 0.5 µM of each primer, and 0.5 U of Taq polymerase (Fermentas). After incubation for 2 min at 94 °C, samples were subjected to 35 cycles of 60 s at the annealing temperature of 38 °C, followed by 1 min at 72 °C; the reaction was completed by 7 min at 72 °C and kept at 4 °C using a PCR-Mastercycler 96 (Eppendorf, Hamburg, Germany). Amplification products were separated on a 1.5% agarose gel stained with ethidium bromide in1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) buffer and photographed.

3.5.4 Results

3.5.4.1 Molecular typing

The result of the molecular typing obtained using the primer $(GTG)_5$ is reported in Fig. 1. The two set of strains, coming from two different ecological niches showed a similar profile with few variations between strains. The profile was characterized by two main bands of 881 and 792 bp, shared by all strains. A third band of 1037 bp was present in the majority of the strains.

Fig 1 REP-PCR profiles of the *K. unispora* strains, with primer $(GTG)_5$



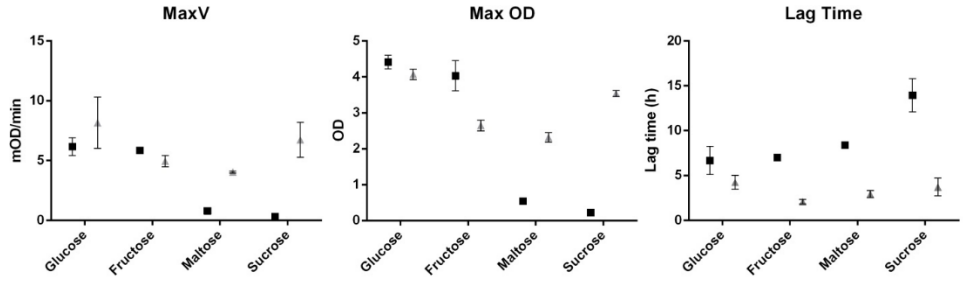
3.5.4.2 Growth and carbon sources utilization

To characterize growth and carbon sources utilization patterns of *K. unispora* strains, the yeasts were cultivated in YP medium supplemented with sugars that are present in flour. The *K. unispora* strains are characterized by rather restricted substrate utilization: only glucose and fructose supported the growth of the strains. Sucrose is not utilized by the strains, indicating a lack of invertase activity.

Regarding glucose and fructose, the growth rate values were similar, although slightly higher in glucose than in fructose. However, the growth in presence of fructose was higher compared to commercial yeast (cell density 31% to 115% higher) for all *K. unispora* strains tested. From the microtiter assay (Fig. 2) it is evident that whereas for *S. cerevisiae* growth rates in glucose, fructose, sucrose and maltose are similar, *K. unispora* grows efficiently in glucose and

fructose only, and although the lag time is higher, the final OD reached is also higher.

Fig. 2 MaxV, Lag time and Max OD of strains with different carbon sources. Results are expressed as mean±SD Δ *S. cerevisiae* SC ■ *K. unispora* KM11



3.5.4.3 Stress tolerance

The viability of the *K. unispora* strains was checked under different stress condition. As reported Fig. 3 the strains could withstand the series of stress and were able of adapting to the conditions tested.

All strains grew well in the pH range 3.0-6.0, whereas at a lower pH, the residual growth compared to the standard YPD medium (pH 6) resulted lower.

In response to temperature change, all the strains were able to grow at temperatures ranging from 25°C to 30°C. At 37°C the residual growth was very low, between 4 and 12% for all strains. No growth was recorded at 45°C after 48 h, furthermore no strain was able to start again the growth when transferred at 28°C.

The presence of ethanol had a great impact on the growth of strains. Moderate growth was observed in presence of 4% and 6% of ethanol, whereas in presence of 12% of ethanol, some level of growth was observed for 44% of the tested strains.

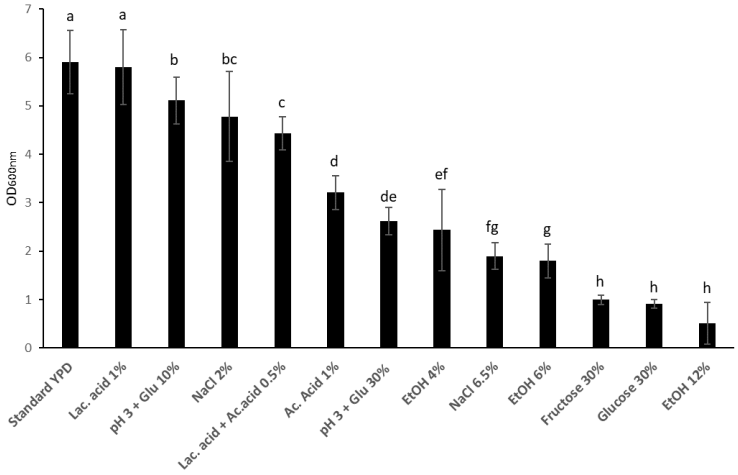
Regarding the tolerance to organic acids, the performance varied when lactic or acetic acid was tested: in presence of 1% of acetic acid, the growth was about 50% of the standard conditions, with few strains showing a better growth

performance, with a 75% of residual growth. Lactic acid did not appear to negatively influence the growth of most strains: for some of them the growth was higher than the standard conditions. The two organic acids did not appear to have any synergistic effect in inhibiting the strains' growth, as they reached intermediate values of growth when compared to the single acids, ranging from 57% to 98% of residual growth.

The strains were also screened for the ability to adapt to osmotic stress, by evaluating the growth in presence of high sugar and salt concentration. In presence of 2% NaCl, a condition similar to concentrations used in bread-making, all strains grew well, with most of them reaching values of 60% of residual growth. A higher concentration (6.5%) reduced the ability of all strains, while still maintaining noteworthy growth levels.

In presence of 30% of glucose or fructose sugars, the growth ranged between 11% and 22% of the standard conditions, which is also due to the fact that in presence of high concentrations of sugars, the metabolism of yeasts shifts to a fermentative one, thus the growth results lower. However, at a pH value of 3, the growth in presence of 30% of glucose was more efficient and was 1.8 to 3.7 times higher than the growth at pH 6.0 and 30% glucose.

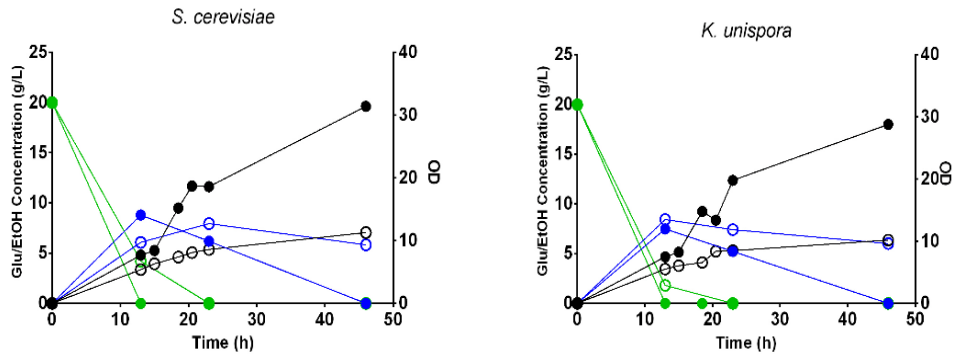
Fig. 3 Stress response of *K. unispora* strains. Results are expressed as mean±SD.



3.5.4.4 Glucose and ethanol assays

One strain of *K. unispora* (KM 11) was used to compare the performance in fermentation with the *S. cerevisiae* strain. The results are reported in Fig. 4. In presence of 20g/L of glucose, the behavior of the two strains is similar: the glucose is rapidly consumed and is not detectable after 13h when the strains were grown in agitating condition, and the ethanol concentration was at a peak level of 8.8 g/L for *S. cerevisiae* and 7.9 g/L for *K. unispora*. In the subsequent hours ethanol was consumed and cell growth continued, until it became undetectable. In static conditions the dynamics were slower; the peak of ethanol production was reached after 23h of fermentation for *S. cerevisiae*, whereas it overlapped with the agitating growth for *K. unispora*. The maximum concentration of ethanol also was comparable between the two conditions, but it was poorly consumed after 46h. The final cell density recorded was comparable for both strains and 3 times lower than that recorded in agitating conditions.

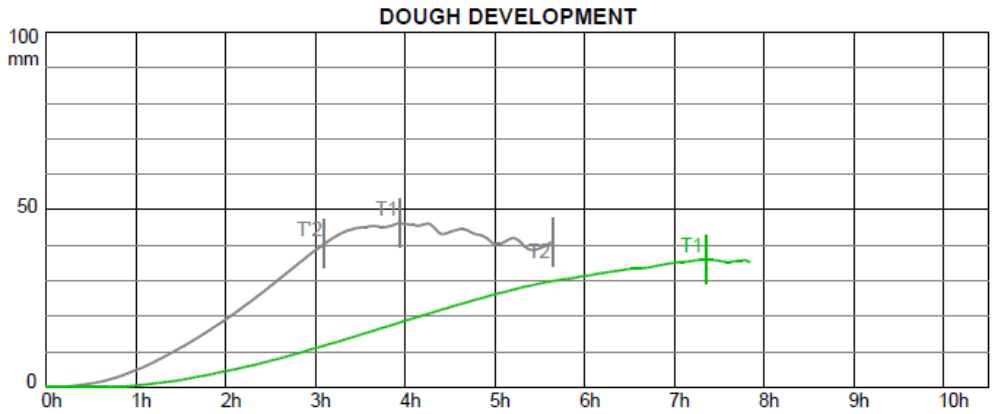
Fig. 4 Glucose consumption, ethanol production and OD of *S. cerevisiae* and *K. unispora* KM11. Full shapes represent agitation conditions, empty shapes represent static growth. ●OD; ● EtOH; ● Glucose



3.5.4.5 Rheofermentometer assay

As expected, due to the inability of the *K. unispora* strain to ferment maltose, the leavening of the dough is slower compared to the commercial *S. cerevisiae* strain, as can be seen in Fig. 5. Whereas *S. cerevisiae* reaches the maximum height after 4 h, the dough leavened with *K. unispora* does not appear to reach the maximum height at the end of the 8h. The height reached by *K. unispora* is 35.9 mm that is lower compared to the 46.2mm reached by *S. cerevisiae*.

Fig. 5 Rheofermentometer analysis. ■ *S. cerevisiae* ■ *K. unispora*



3.5.5 Discussion

In this study we tested some properties not yet known of *K. unispora* strains, with the aim to evaluate their potential role as non-conventional yeast species in sourdough fermentation. Nowadays, there is an increasing interest of revisiting the starter cultures, including the autochthonous microbial population, in an attempt to improve the peculiar characteristics, the quality and the safety of the final products. In this context, it is important to explore and to study the potential of new strains from diverse ecological niches for industrial relevant uses.

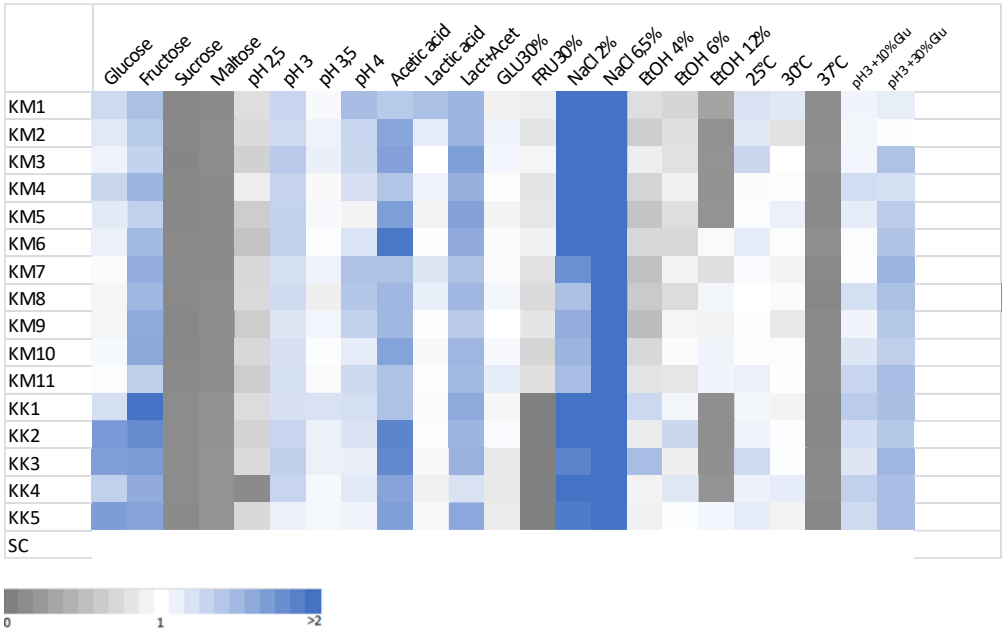
The genus *Kazachstania*, the closest neighbour to the genus *Saccharomyces*, comprises several species that can be found in diverse habitats, among which fermented food (Kabisch et al. 2013; James et al. 2015; Jacques et al. 2016). Particularly, *K. unispora* plays a significant role in both the ripening of cheese and in the production of fermented milk products such as a kefir and koumiss (Mu et al. 2012; Bourrie et al. 2016). Moreover, *K. unispora* seems to contribute to aroma development during the wine fermentation even if this species is encountered at low

frequency in grape must (Jodd et al. 2017). This species has also been documented in fermented orange fruit and juice (Bhattacharya et al. 2013) and in sourdough ecosystems, especially in North American and European sourdoughs (Lhomme et al. 2016; De Vuyst et al. 2016). However, despite its relevant presence in different food niches, the role and functions of this species is poorly known. In relation to the backing sector, together with the industrial bread production obtained using the commercial baker's yeast *Saccharomyces cerevisiae,* commercially sourdoughs containing selected yeasts and LAB strains are also available. If the LAB proposed as sourdough starters are well defined, the search for non-conventional yeast species is still in progress. In this context, we deepened the knowledge of several strains of *K. unispora.*

Our results show that *K. unispora* are adapted to a sourdough environment, since the strains tested are able to grow in conditions associated with it, such

as high osmolarity, high acidity and the presence of organic acids, ethanol and salt. Moreover, when the performance of the *K. unispora* strains was compared to the commercial strain of *S. cerevisiae* (Fig. 6) it was possible to note that strains of *K. unispora* performed better under some stress conditions, in particular in high salinity or in presence of acetic acid, with all strains outgrowing *S. cerevisiae*; the commercial strain grew very poorly in the medium added with 6% NaCl and showed a moderate growth in presence of acetic acid. The tolerance to low pH values is similar for all strains, whereas at pH 4 (the pH value typical of type I sourdoughs) the growth is more efficient for *K. unispora* strains. In combined pH and osmolarity conditions, almost all *K. unispora* strains outgrow the *S. cerevisiae* strain. On the contrary, *S. cerevisiae* is able to resist to high ethanol concentrations, outgrowing most *K. unispora* strains, although some strains achieved a comparable growth even at 12% of ethanol. *S. cerevisiae* performed better when grown in sucrose and maltose, due to the inability of the strains of *K. unispora* to utilize these carbon sources, as well as at 37°C. However, strains of *K. unispora* reached a higher cell density when grown in glucose and fructose compared to *S. cerevisiae* grown in similar conditions. The *K. unispora* strains are maltose-negative, but this characteristic might be not negative in sourdoughs: their inability to ferment maltose makes them suitable strains in stable consortia with maltose consuming LAB, which supply the fermentable sugars used by the yeast in fermentation (Gobbetti 1998; De Vuyst et al. 2009; Carbonetto et al. 2020). *K. unispora* strains are also unable to ferment sucrose. This characteristic is not advantageous when molasses are used for the industrial production of baking yeast starter cultures. The cost and efficient use of carbon sources are crucial for economical production of yeast biomass. However, alternative low-cost substrates could be used for *K. unispora*, such as glucose syrups derived from starch hydrolysis, high fructose corn syrups (HFCS) or by-product of corn-starch extraction process (Spigno et al. 2009; Yu et al. 2015).

Fig 6. Heatmap of the performance of each *K. unispora* strain relative to *S. cerevisiae* in stress conditions. Scale shows the proportion of growth of *K. unispora* compared to *S. cerevisiae*: 0 indicates no growth, 1 indicates equal growth, >2 indicates the growth is at least twice as much.



The performance in fermentation was inferior compared to *S. cerevisiae*, but the growth was more efficient, which is an advantage in utilizing these strains in an industrial scale. Moreover, although *K. unispora* is outperformed in a straight dough context, its longer fermenting time and the ability to act in synergy with LAB can be used in sourdough production, where, especially for a type I sourdough, longer leavening times are typical. For these reasons, it is possible to hypothesize the creation of mixed cultures consisting of and *K. unispora* and selected LAB strains for sourdough bread making. Further experiments are in progress in our laboratory, testing this microbial association and evaluating the characteristics they provide to the final bread product.

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3.6 Technological properties, shelf life and consumer preference of spelt-based sourdough bread using novel, selected starter cultures.

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3.6.1 Abstract: The goal of this work was to investigate the use of selected starter cultures to obtain a spelt-based sourdough bread with improved technological, sensory and shelf-life characteristics. Two consortia were set up, containing a yeast strain (either a commercial *Saccharomyces cerevisiae* strain or a maltose-negative *Kazachstania unispora* strain) and two strains of Lactic Acid Bacteria (LAB), belonging to *Weissella cibaria* and *Pediococcus pentosaceus* species. The ability to grow in co-culture was investigated, and no inhibitions were recorded between the LAB and yeasts, that grew in proportions deemed desirable for sourdoughs. The performance of the two consortia was assessed in a spelt-based sourdough bread, and the leavening behavior, bread volume and crumb softness, shelf life and consumer preference were assessed. The product obtained with the consortium containing *S. cerevisiae* had better crumb texture that was maintained through 5 days of storage, and was well accepted by the consumers. Furthermore, both consortia improved the mold free shelf-life when challenged with common cereal contaminants. The data presented show that selected starter cultures have a good potential in improving the quality of bakery products obtained with flours that have a poor technological performance, such as spelt, but interesting nutritional properties and sustainable cultivation.

3.6.2 Introduction

Sourdough fermentation for bread production is one of the earliest uses of bioprocesses, dating back thousands of years ago, and remains to this day one of the staple components of the human diet. Although the direct method is often used in bread production at industrial levels, the sourdough technology is still commonplace in artisanal bakeries. This practice offers a series of advantages, as several studies show the beneficial activity of Lactic Acid Bacteria (LAB) and yeasts in the production of bioactive compounds (Katina et al., 2005, Poutanen, Flander & Katina, 2009), removal of antinutrients, such as phytate (De Angelis et al., 2003; Leenhardt, Levrat-Verny, Chanliaud, & Rémésy, 2005; Lopez et al., 2001) and improvement in texture, taste and shelf life of bread (Arendt, Ryan, & Dal Bello, 2007; Dal Bello et al., 2007; Katina, Salmenkallio-Marttila, Partanen, Forssell, & Autio, 2006).

Sourdough is a mixture of flour and water, fermented with autochthonous LAB that contribute to the aroma, taste and technological properties of the product by producing organic acids such as lactic and acetic acid. The downside of using sourdough in larger scales is the fact that the microbial composition of the sourdough may vary due to several factors, such as the time and temperature of fermentation or the chemical and microbial composition of the flour, making sourdough a difficult ingredient to standardize. A solution to this problem may be the use of well-defined starter cultures that have been selected for their desirable activities and stability. The use of sourdough is a traditional practice in many areas of the world, including Central and Eastern Europe and Scandinavia, where sourdough breads with mixed flours containing rye, barley or wheat are commonly consumed (Hammes & Gänzle, 1998). For rye, acidification achieved either chemically, or, principally, through sourdough fermentation is an essential step, that allows obtaining a desirable texture. The gluten network in rye dough is very weak, so acidification promotes water absorption by pentosans, as well as inhibits the activity of rye amylases, that can reduce loaf volume (Hammes & Gänzle, 1998; Weckx et

al., 2010). Similarly, sourdough has been successfully used in gluten-free bread to improve the texture and flavor (Schober, Bean, & Boyle, 2007; Wolter, Hager, Zannini, Czerny, & Arendt, 2014).

Spelt (*T. aestivum* ssp. *spelta* (L.) is one of the oldest cereals, an ancient grain which, after decades of marginal cultivation, has been upgraded for its reputation as a healthy and sustainable food. When compared to wheat (*T. aestivum* ssp. *aestivum*), spelt shows a higher content in proteins, soluble fiber and micronutrients, and lower levels of fermentable oligo- di- and monosaccharides and polyols (FODMAPs) that can lead to gastrointestinal symptoms after fermentation by the gut microbiota, as well as a better aminoacidic and lipidic profile (Escarnot, Jacquemin, Agneessens, & Paquot, 2012; Frakolaki, Giannou, Topakas, & Tzia, 2018). Furthermore, spelt needs less inputs for its cultivation, such as pesticides and fertilizers, and can grow on marginal lands and on poorly drained and low-fertility soils, so it is a highly sustainable crop. Unfortunately, the dough obtained with spelt flour has a low elasticity, resulting in a decreased volume and increased firmness of the final product (Frakolaki, Giannou, & Tzia, 2020). Mixing spelt and wheat flour could be a valid compromise to give the bread a greater nutritional contribution, and an acceptable quality. Furthermore, the use of a microbial association consisting of LAB and yeasts, already adapted to the cereal environment, could better contribute to the rheological properties of the dough.

In this study two novel microbial consortia were set up and used to obtain spelt-based sourdough bread. The aim of this research was to investigate the activity of the mixed cultures in a guided sourdough fermentation on the technological performance, stability, consumer acceptability, and shelf life of the spelt-based bread.

3.6.3 Materials and methods

3.6.3.1 Strains and growth conditions

Two selected strains of Lactic Acid Bacteria (LAB) belonging to the species *Pediococcus pentosaceus* (strain MB33) and *Weissella cibaria* (strain CM32), and one yeast strain belonging to *Kazachstania unispora* species (strain KM11), previously isolated from natural cereal fermentations (Decimo et al., 2017; Korcari, Ricci, Quattrini, & Fortina, 2019), were studied in comparison with a *Saccharomyces cerevisiae* strain (strain SC) obtained from a commercial baker's yeast preparation. The LAB were routinely sub-cultured in MRS broth/agar (Difco Lab., Augsburg, Germany) medium for 24-48 h at 30° C, whereas the yeasts were grown in YPD broth in the same conditions. The composition of the YPD medium is as follows (g/L): Yeast extract (10), Peptone (20), Glucose (20), pH 6.2.

All strains were deposited in the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy, at −80° C in MRS for LAB and YPD for yeasts with 15% glycerol.

Representatives of common fungal spoilage of bread, *Aspergillus niger*, *A. flavus* and *Fusarium verticillioides* (from the Collection of the Department of Health, Animal Science and Food Safety, University of Milan, Italy) were used as target strains for the antifungal assay. Fungal strains were grown on malt extract agar (MEA) (Merck, Darmstadt, Germany) at 25° C for 5–7 days and spore suspensions were harvested by adding 15 mL of sterile Milli-Q water and counted by flow cytometer estimation (BD Accuri C6 Flow Cytometer, BD Biosciences, Franklin Lakes, USA).

3.6.3.2 Co-cultures and microbial composition analyses

In order to study the stability of the consortia SCLAB (*S. cerevisiae* SC, *W. cibaria* CM32, *P. pentosaceus* MB33) and KULAB (*K. unispora* KM11, *W. cibaria* CM32, *P. pentosaceus* MB33), mixed cultures were grown either in MRS broth or doughs. The total yeast and LAB counts were performed in

triplicate in YGC and MRS agar plates, respectively. For evaluating the growth in doughs of the two different LAB strains, a qPCR experiment was set up. For this purpose, doughs prepared with spelt flour were inoculated with serial dilutions of an overnight-grown broth for each bacterium in a concentration range of 3-8 log cycles. For DNA extraction 1 mL of the 1:10 diluted dough sample was centrifuged at 500 rpm for 1 min, the supernatant was recovered and centrifuged at 15000 rpm for 5 min to recover the cellular pellet, which was then used for the phenol-chloroform total DNA extraction. The DNA obtained was used for the standard curve determination. The primers and thermal cycles are reported in Table 1. The PCR reaction was carried out in a total volume of 15 µL, containing 7.5 µL of qPCR mix (SSO Fast Supermix, BioRad, Hercules, USA), 0.36 µL of each primer (0.3 µM), 1.78 µL of PCR grade water and 5 µL of DNA. The threshold level was set by the instrument (LineGene 9600 series, Bioer technology, Hangzhou, China), and the efficiency was calculated with the formula $E= 10^{(1-/slope)} -1$ (Rutledge & Cote, 2003). The efficiency was deemed acceptable if it fell in the range 90-110%.

Table 1 Primers and thermal cycles for qPCR evaluation of *Pediococcus* and *Weissella.*

Primer	Sequence	Thermal cycles	Reference
Pediococcus spp.	F: GAACTCGTGTACGTTGAAAAGTG CTGA R: GCGTCCCTCCATTGTTCAAACAAG	94°C x 20s 66°C x 20s 72°C x 40s	Pfannebecker & Fröhlich (2008)
Weissella spp.	F: CGTGGGAAACCTACCTCTTA R: CCCTCAAACATCTAGCAC	94°C x 20s 54°C x 20s 72°C x 40s	Jang et al. (2002)

3.6.3.3 Sourdough fermentation and bread preparation

Sourdoughs were prepared by mixing 100% spelt flour (protein content: 13%; fiber content: 8%; Molino Quaglia S.p.A., Padua, Italy) and 52% sterile tap water, inoculated with different microbial consortia (Table 2). Each sample was mixed for 4 min and incubated at 30° C for 16 h. Sourdough bread was prepared by mixing a 1:1 mixture of wheat and spelt flour, 63% of tap water (27° C), 30% of sourdough and 1.5% salt (all percentages are flour basis). The technological performance of the two consortia SCLAB and KULAB were compared to a control dough prepared by inoculating *S. cerevisiae* only in a dough prepared using exclusively refined wheat flour (00D; Molini Lario S.p.A.; protein: 9%; W: 190-210*10^{-4} J; P/L: 0.6-0.8). The doughs obtained were kneaded for 5 min, maintained at rest for 10 min, divided into 250 g pieces and put into baking pans for proofing at 30° C according to the rheofermentographic test, and finally baked at 190° C for 18 min, in presence of steam during first stages of baking. After baking, the bread samples were cooled to 20° C and samples were taken for further analyses.

Table 2. Microbial consortia inoculated in spelt sourdough.

Sourdough samples	Consortia composition	Cell density (log_{10}CFU/g)
SC	*Saccharomyces cerevisiae*	6
SCLAB	*S. cerevisiae* *Pediococcus pentosaceus* *Weissella cibaria*	6 6 5
KU	*Kazachstania unispora*	6
KULAB	*K. unispora* *P. pentosaceus* *W. cibaria*	6 6 5

3.6.3.4 Dough leavening properties and bread features

The leavening properties of the consortia were evaluated on 315 g of dough, prepared in the conditions reported in the previous section, by means of the Rheofermentometer F4 (Chopin, Tripette & Renaud, Villeneuve La Garenne Cedex, France), recording changes in dough height and production and retention of CO_2 during fermentation at 30° C for 6 h.

The specific volume of loaves was measured in triplicate by the ratio between the apparent volume (AACC 10-05.01; AACC 2001) and its mass. In addition, the crumb and crust color were determined using a reflectance color meter (CR 210, Minolta Co., Osaka, Japan), and the results were expressed in the CIELAB color space (L*, a*, b*), as the mean ±SEM of 4 evaluations. Crumb moisture and its water activity (a_w) were determined in two separate measurements by means of the Moisture Tester MT-CA (Brabender GmbH&Co KG, Duisburg, Germany) at 130° C for 1 h, and by a hygrometer (Novasina AG, Zurich, Switzerland) at 25° C, respectively. At the end, crumb texture was measured in triplicate by using a Texture Analyzer TA.XT plus C (Stable Micro Systems, Surrey, UK), after 1, 2 and 5 days of storage, according to the AACC official method (AACC 74–09.01; AACC 2001).

3.6.3.5 Antifungal activity challenge test

To test the in situ antifungal activity, a challenge test as described by Black, Zannini, Curtis, & Gänzle, (2013) was performed. 25 mm slices of each bread sample were inoculated with 10^2 spores of *A. niger*, *A. flavus* and *F. verticillioides*, stored in closed plastic bags with a filtered tip to ensure aerobic conditions, at room temperature for up to 7 days. The growth of the molds was recorded daily. The shelf life was expressed as the number of days before visible mold growth.

3.6.3.6 Assessment of consumer's acceptability

The acceptability of the four different types of spelt-based bread was assessed involving 86 regular consumers of bread (48 females and 38 males;

mean age = 26.4 years; s.d. = 8.1) recruited among students and staff of the University of Milan. Although the number of subjects is somewhat small, it fulfills the requirements to perform a hedonic test (ISO 11136, 2014).
Participants were asked not to smoke, eat or drink anything, except water, for 1 h before the tasting session. The protocol was approved by the Ethics Committee of the University of Milan. Written informed consent was obtained from each subject before the acceptability assessment was performed.
Subjects were invited to the sensory laboratory of the Department of Food, Nutritional and Environmental Sciences of the University of Milan and were settled in individual sensory booths.
Subjects were presented with a slice of bread for each sample and asked to express their overall liking using a 100-mm linear hedonic scale anchored at the extremes with "dislike extremely" (left of the scale, score = 0) and "like extremely" (right of the scale, score = 100). Subjects were instructed to taste a piece of each slice and to rinse their mouth with mineral water between each tasting. The evaluation took approximately 10 minutes.
In order to balance the effects of serving order and carry-over, samples presentation order was randomized and balanced according to William's Latin square (Macfie, Bratchell, Greenhoff, & Vallis 1989). Samples were served at room temperature (about 20° C) in plastic plates coded with 3-digit numbers and evaluated under white light conditions. The sample SCW (refined wheat bread inoculated with *S. cerevisiae* only) was omitted because preliminary results showed the difference this bread had with the other samples could lead the participants to underestimate the differences between the spelt-based samples.

3.6.3.7 Statistical analysis

Results are expressed as mean value ± SEM and paired comparisons were analyzed with two-tailed t-test (asterisks indicate significance levels: *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s. for $p > 0.05$). Sensory data were normally distributed ($W = 0.986$, $p = 0.467$) and analyzed through mixed Analysis of Variance

(ANOVA) considering subjects as a random effect and bread samples as fixed effect. Tukey's HSD test was performed after the ANOVA using XLSTAT (version 2020.5.1, AddinsoftTM, France).

Effects showing a p-value of 0.05 or lower were considered significant.

3.6.4 Results

3.6.4.1 Strains selection and co-culture growth

The microbial strains used in this research were previously selected in relation to useful physiological properties. Specifically, the strain CM32 of *W. cibaria* was chosen based on previous screenings (Quattrini, Korcari, Ricci, & Fortina, 2019) for its ability of producing exopolysaccharides, a high β-xylosidase activity, a high redox potential as well as the ability to inhibit the growth of *Fusarium verticillioides* and *Aspergillus flavus*. The strain *P. pentosaceus* MB33 was also chosen for the antifungal activity towards *Mucor circinelloides*, *A. flavus* and *F. verticillioides* and a good acidifying ability (Korcari, Ricci, Quattrini, & Fortina, 2019). *K. unispora* KM11 was chosen as an alternative yeast species; in previous researches (Korcari, Ricci, Capusoni, & Fortina, 2021) the strain showed a good leavening performance and a maltose-negative phenotype that may be advantageous in stable consortia with maltose consuming LAB (De Vuyst & Neyens, 2005).

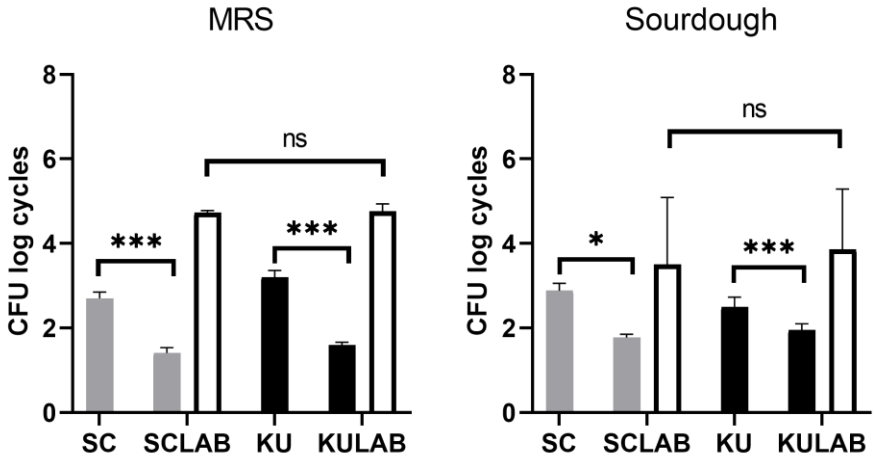
S. cerevisiae and *K. unispora* showed a similar growth when cultivated alone or in presence of LAB. The growth in co-culture in MRS medium showed limited inhibition in 16 h of incubation (Fig 1). The reduced growth of the yeasts when grown in combination with *P. pentosaceus* could be probably due to the limited nutrients in the medium, rather than inhibition, whereas the heterofermentative growth of the *W. cibaria* strain, producing different organic acids, could affect the growth of the yeasts; indeed, previous research showed that both yeast strains grow efficiently in presence of lactic acid, but the growth is limited in presence of acetic acid (Korcari, Ricci, Capusoni, & Fortina, 2021). The growth of the LAB was unaffected by the presence of the yeast: the recorded growth ranged between 4.56 and 4.91 log cycles in all conditions.

Similar results were obtained when the strains were inoculated in spelt dough: the growth of the yeasts was efficient but was slightly inhibited by the LAB: whereas when inoculated alone *K. unispora* grew 2.5 log cycles, in presence of the LAB the growth resulted being 1.95 log cycles, the growth of *S.*

cerevisiae was reduced from 2.88 log cycles when inoculated alone to 1.77 log cycles when used in combination with the LAB. However, the proportion between yeast cells and LAB at the end of the fermentation was 1:100, that is considered optimal for sourdough preparation. Despite the inhibitory activity of acetic acid, the presence of the *W. cibaria* strain was deemed necessary to achieve the typical flavor and characteristics of sourdough bread, where heterofermentative LAB are play a major role.

The qPCR analysis showed that the LAB grew in a similar way independently of the yeast used. During the sourdough fermentation *W. cibaria* outgrew *P. pentosaceus*, in a 4:1 proportion. When this sourdough was used to obtain a leavened dough, *P. pentosaceus* grew more efficiently, and the proportion between the two LAB was closer to 1:1.

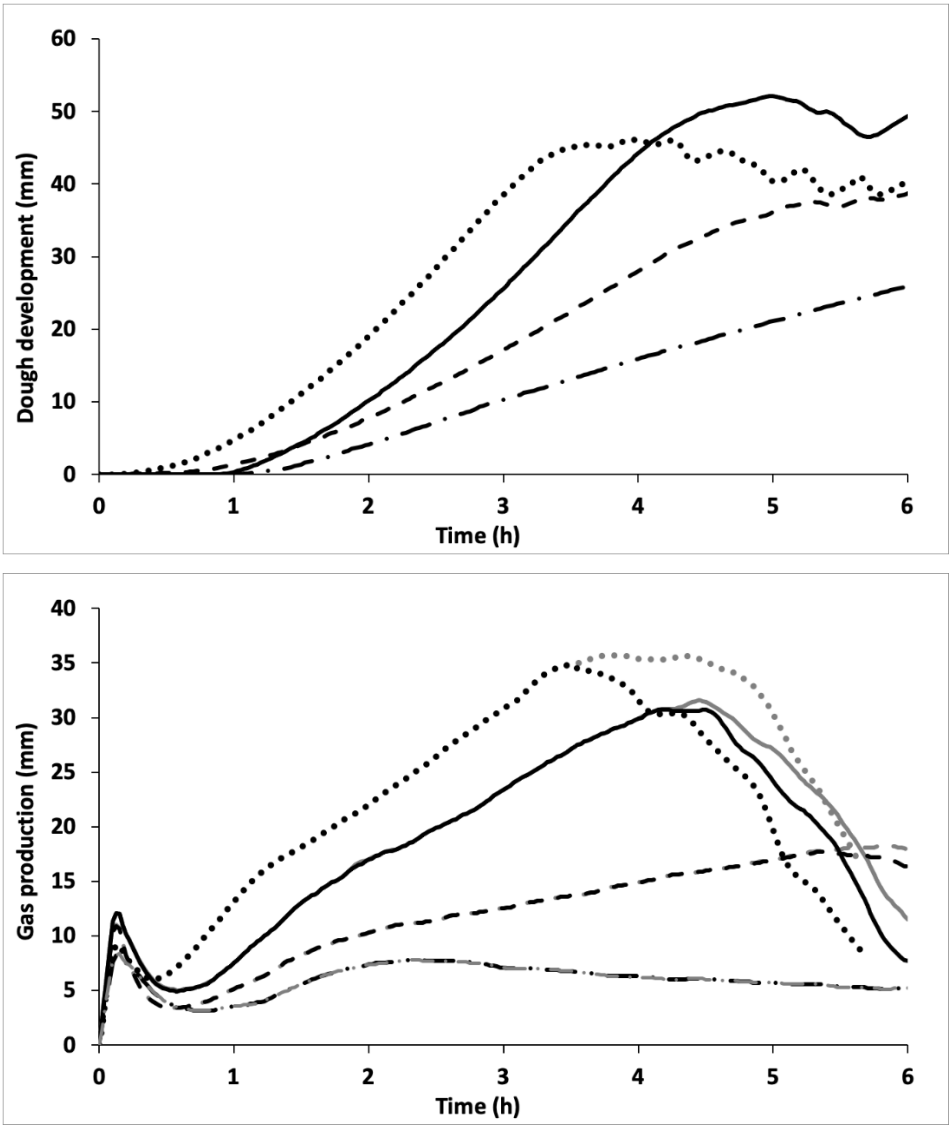
Fig. 1. Growth in MRS and in spelt sourdough of yeast strains alone or in association with LAB strains (two tailed t-test, asterisks indicate significance levels: *p≤0.05; ***p≤0.001) SC: *S. cerevisiae*; SCLAB: consortia of *S. cerevisiae*, *P. pentosaceus* and *W. cibaria*; KU: *K. unispora*; KULAB: consortia of *K. unispora*, *P. pentosaceus* and *W. cibaria*. Mean±SEM (n=3)



3.6.4.2 Dough leavening properties

Compared to wheat dough, the presence of spelt in the formulation decreased, even though slightly, both the maximum dough height (52 *vs* 46 mm for SCW and SC, respectively) and the time to reach it (~5 *vs* ~4 h for SCW and SC, respectively) (Fig. 2). As regards gas production, the presence of spelt increased both the CO_2 produced (~1086 *vs* ~1275 mL for SCW and SC, respectively) and that one released (~43 *vs* ~133 mL for SCW and SC, respectively). When LAB were used together with *S. cerevisiae* the resulting dough required a longer time (~6 h) to reach the dough maximum height (~39 mm), but the volume of gas produced (675 mL) and released (6 mL) decreased. The worsening of leavening dough performance was even more pronounced when the *K. unispora* strain was used instead of SC. Indeed, the consortia *K. unispora* and the LAB after 6 h of fermentation reached a dough maximum height of about 27 mm and the amount of CO_2 produced was about 415 mL.

Fig. 2. Effects of SCW (solid lines), SC (dotted lines), SCLAB (dash lines), KULAB (dash-dot lines) on (a) dough development and on (b) gas production (grey lines) and retention (black lines). SCW: *S. cerevisiae* in wheat sourdough; SC: *S. cerevisiae* in spelt sourdough; SCLAB: consortia of *S. cerevisiae*, *P. pentosaceus* and *W. cibaria* in spelt sourdough; KULAB: consortia of *K. unispora*, *P. pentosaceus* and *W. cibaria* in spelt sourdough.



3.6.4.3 Bread quality

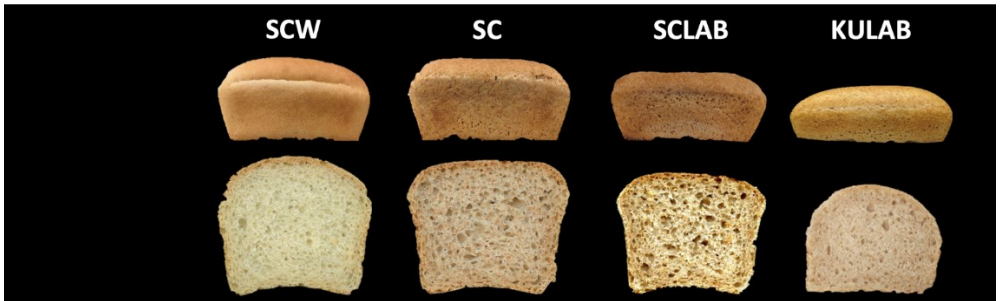
The presence of spelt flour did not cause significant ($p>0.05$) changes in terms of either specific volume (Fig. 3) and crumb softness (Fig. 4) compared to bread produced by refined wheat flour alone. On the other hand, the combination between *S. cerevisiae* and LAB led to a decrease in specific volume (-10%). The decrease in specific volume was more evident (-30%) when *S. cerevisiae* was replaced with *K. unispora* in combination with LAB.

As regards the crumb firmness, the presence of LAB decreased this parameter up to 48 h of storage, when compared to bread leavened with *S. cerevisiae* only (Fig. 4). Based on this parameter the worst performing consortium was *K. unispora* and the LAB, as the sample reached the highest crumb firmness already after 24 h of storage.

The relative humidity and water activity of the samples did not differ significantly between the samples, and decreased as the storage time increased (data not shown). For this reason, the differences in crumb firmness between the samples are more likely due to the different leavening ability of the strains used.

From the color analysis performed on the samples (Fig. 3), it emerged that the presence of spelt, with the same leavening agent used (i.e., *S. cerevisiae*), did not significantly affect the crust color of the bread. In contrast, the related crumb became darker (i.e., decrease in L* index) and redder (i.e., increase in a* index) when spelt was used, while no change was observed in terms of yellowness (b* index). In addition, when the LAB were added, the crust resulted both less red and yellow. Unlike the consortia composed of *S. cerevisiae* and LAB, the crust of the sample containing *K. unispora* and the LAB did not differ significantly from the latter.

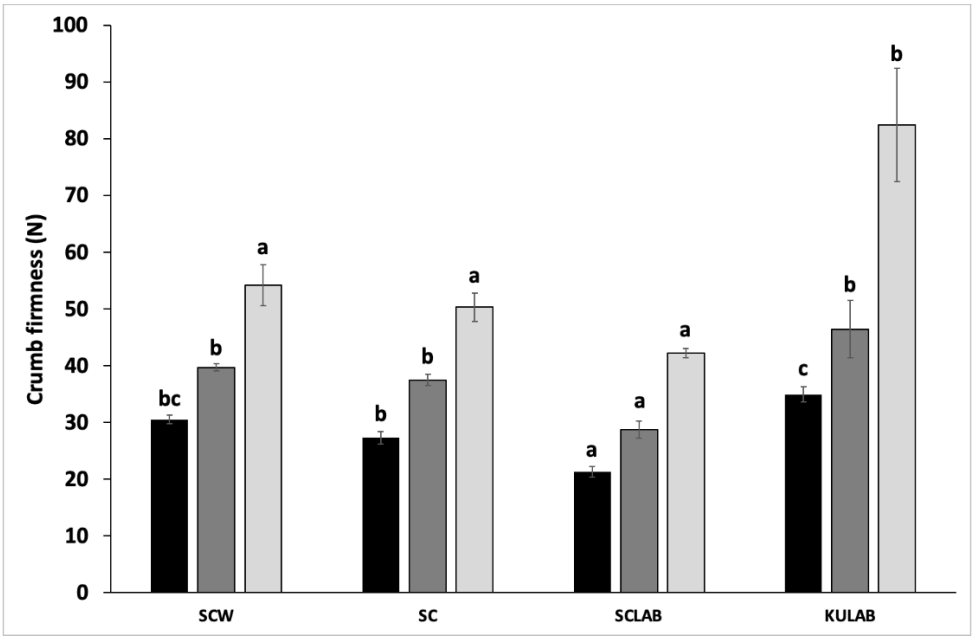
Fig. 3. Bread and slices of bread, bread volume and specific volume and crumb and crust color. SCW: *S. cerevisiae* in wheat sourdough; SC: *S. cerevisiae* in spelt sourdough; SCLAB: consortia of *S. cerevisiae*, *P. pentosaceus* and *W. cibaria* in spelt sourdough; KULAB: consortia of *K. unispora, P. pentosaceus* and *W. cibaria* in spelt sourdough. Different letters in the same row indicate significant differences (Tukey's HSD test, $p<0.05$). Mean±SEM (n=3 for volume and specific volume; n=4 for crust and bread colour).



		SCW	SC	SCLAB	KULAB
BREAD	Volume (mL)	605±3^{c}	617±3^{c}	550±10^{b}	437±7^{a}
	Specific volume (g/mL)	2.91±0.01^{c}	3.02±0.01^{c}	2.71±0.04^{b}	2.14±0.04^{a}
CRUST	Luminosity (L*)	57±4^{ab}	53±2^{a}	58±1^{ab}	63.2±0.7^{b}
	Redness (a*)	21±2^{ab}	24±1^{b}	17.7±0.7^{a}	20.6±0.4^{ab}
	Yellowness (b*)	40±4^{b}	40±1^{b}	34.6±0.5^{a}	35.3±0.8^{a}
CRUMB	Luminosity (L*)	80±1^{b}	63±2^{a}	62.7±0.4^{a}	56.7±0.6^{a}
	Redness (a*)	-1.8±0.1^{a}	4±1^{b}	7.9±0.2^{c}	6.7±0.2^{c}
	Yellowness (b*)	22.3±0.2^{b}	22±1^{b}	37.9±0.2^{c}	13.0±0.3^{a}

As regards crumb color, regardless of the leavening agent, the luminosity did not differ significantly among the samples containing spelt flour. The presence of LAB led to an increase in crumb redness compared to the sample containing *S. cerevisiae* only, whereas a different trend was observed in terms of yellowness. Indeed, this index significantly increased and decreased when LAB were used together *S. cerevisiae* and *K. unispora*, respectively (Fig. 3).

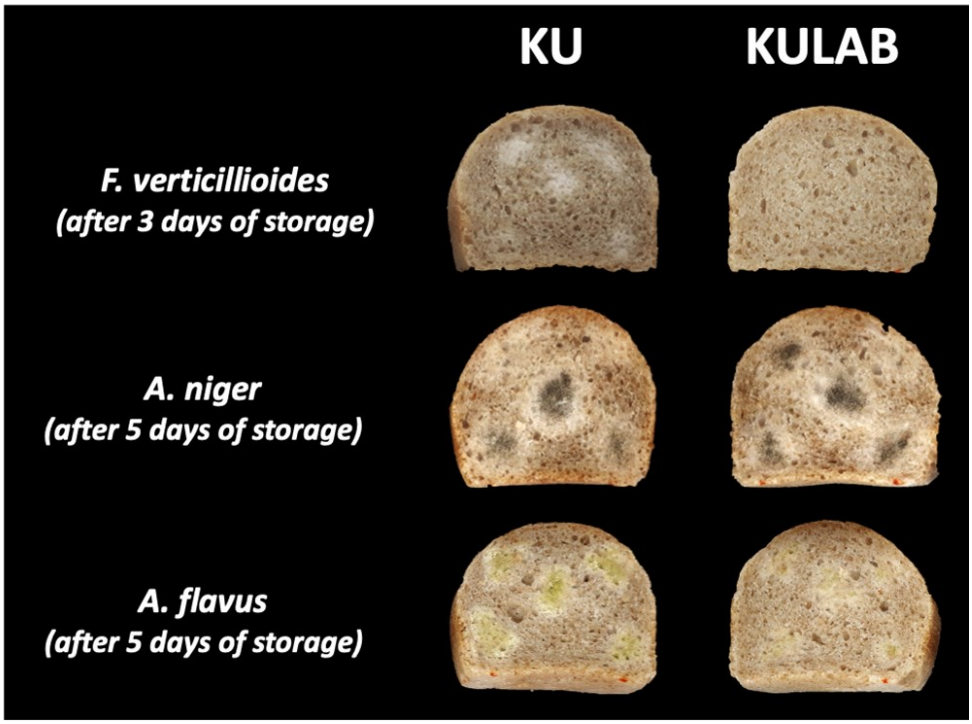
Fig 4: Crumb firmness (N) after 1 (black bars), 2 (dark grey bars) and 5 (light grey bars) days of storage. SCW: *S. cerevisiae* in wheat sourdough; SC: *S. cerevisiae* in spelt sourdough; SCLAB: consortia of *S. cerevisiae*, *P. pentosaceus* and *W. cibaria* in spelt sourdough; KULAB consortia of *K. unispora, P. pentosaceus* and *W. cibaria* in spelt sourdough. Different letters in the same day indicate significant differences (Tukey's HSD test, $p<0.05$). Mean±SEM (n=3).



3.6.4.4 Antifungal activity

The antifungal activity of the consortia towards common contaminants of cereals and bakery products was assessed, after preliminary indications of an *in-vitro* antifungal activity exerted by the two strains of LAB used (Quattrini, Korcari, Ricci, & Fortina, 2019). The challenge study showed that the consortia containing the LAB, independently of the yeast used, extended the shelf life of the bread inoculated with *F. verticillioides* by 24 h, as well as inhibited the growth and sporification of the sample inoculated with *A. flavus* (Fig. 5). No inhibition was exerted towards *A. niger*.

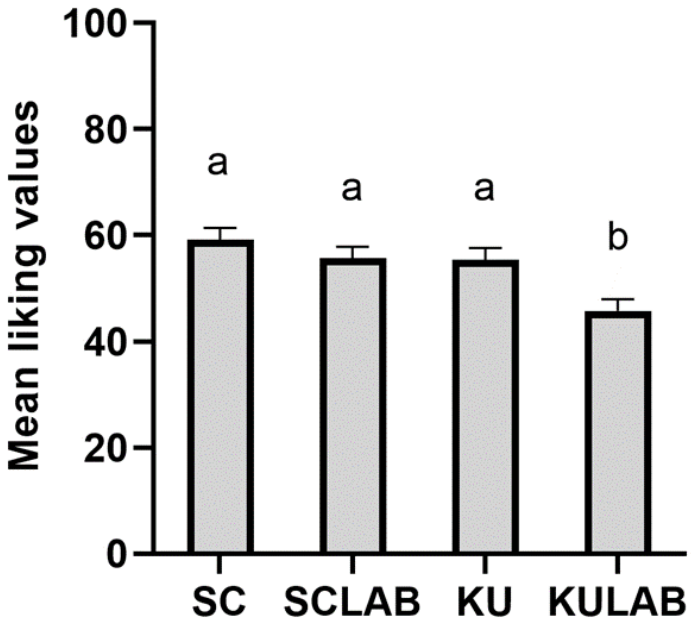
Fig. 5. Antifungal activity assay of bread obtained with *K. unispora* strain alone or in association with LAB.



3.6.4.5 Consumer's acceptability

The mean liking scores of the bread samples are shown in Fig. 6. Bread samples were significantly different in terms of acceptability ($F_{(3,255)}$= 11.1, $p < 0.001$). With the exception of the *K. unispora* in association with LAB, all bread samples were scored higher than the 50% of the hedonic scale (corresponding to "neither liked nor disliked"). Post-hoc comparison indicated that the *S. cerevisiae* in association with LAB and *K. unispora* samples received an acceptability score that was statistically comparable to the sample SC, whereas the sample obtained with *K. unispora* in association with LAB was statistically different and significantly less liked than the other samples.

Fig. 6. Mean acceptability scores with SEM of the different bread samples. Different letters indicate significant differences ($p<0.05$) according to post-hoc Tukey's HSD test. SC: *S. cerevisiae* in spelt sourdough; SCLAB: consortia of *S. cerevisiae*, *P. pentosaceus* and *W. cibaria* in spelt sourdough; KU: *K. unispora* in spelt sourdough; KULAB: consortia of *K. unispora, P. pentosaceus* and *W. cibaria* in spelt sourdough.



3.6.5 Discussion

Sourdough fermentation is an ancient use of biotechnology that is gaining interest in the recent years due to the positive effects on the structure, taste, and shelf life of baked products, thanks to the activity of the LAB and yeasts (Rehman, Paterson, & Piggott, 2006). The increased demand for specialty products such as baked goods obtained from ancient grains, and the nutritional and environmental benefits that these grains have made them an important ingredient that meets both consumers' and industries' demands (Gosine & McSweeney, 2019; Kraska, Andruszczak, Gawlik-Dziki, Dziki, & Kwiecińska-Poppe, 2020; Teuber, Dolgopolova, & Nordström, 2016).

The use of selected cultures, already adapted to the specific environment, has a great potential for obtaining a product with improved quality in a controlled manner, that is a desirable feature at an industrial level. This work showed that this approach is a viable option in obtaining baked products from flours with low baking performances, such as spelt.

The ability to grow in co-culture is essential for the stability of the starter cultures, and our experiments showed that *S. cerevisiae* was only slightly inhibited by the heterofermentative *W. cibaria*, and *P. pentosaceus*. These LAB species are generally considered to be only secondary to sourdough fermentation, dominating the first steps of sourdough backslopping, as species of the old *Lactobacillus* genus, especially *Fructilactobacillus sanfranciscensis*, *Levilactobacillus brevis, Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* dominate the sourdough environment (Oshiro, Zendo, & Nakayama, 2021). However, the positive characteristics highlighted in this research show that these alternative species are interesting and should be considered as adequate starters for sourdough fermentation. Specifically, the addition of *W. cibaria* and *P. pentosaceus* strains extended the shelf life, in terms of delay in fungal growth, of the spelt-enriched bread. As regards the technological properties of bread, regardless of the flour type (wheat flour alone or in presence of spelt), the use of *S. cerevisiae* alone led to the highest bread volume (Fig. 3). Combining LAB with *S. cerevisiae* decreased the dough

development capability, as a result of the decrease in CO_2 production. The lower specific volume of loaves leavened by *S. cerevisiae* and LAB compared to those produced only with *S. cerevisiae* agrees with the findings by other authors (Bottani et al., 2018; Pagani, Lucisano, & Mariotti, 2008). On the contrary, other authors reported the ability of sourdough to improve the volume of the resulted bread in comparison with bread leavened by yeast only (Clarke, Schober, Angst, & Arendt, 2003, Corsetti et al., 2000). These different results might be related not only to the different leavening properties of the microorganism used, but also to the baking condition applied (e.g., temperature and leavening time).

Despite the decrease in volume, the presence of LAB enhanced the crumb softness. Such positive effect was not related to the moisture content, since no differences in this index were found among the bread samples (data not shown). Also, Novotni et al. (2012) found lower crumb firmness when sourdough fermentation was used instead of yeast. According to Katina, Heiniö, Autio, & Poutanen (2006), the softer crumb texture of sourdough bread might be attributed to its lower pH compared to that leavened by *S. cerevisiae*. Previous studies showed that the presence of organic acids might cause a significant decrease in specific volume as consequence of weakening of starch and protein structure (Galal, Varriano-Marston, & Johnson, 1978; Takeda, Matsumura, & Shimizu, 2001).

The decrease in crumb luminosity after the addition of spelt flour compared to bread obtained from common wheat flour alone, could be explained by addition of spelt wholegrain flour, containing dark bran particles. The increase in crumb darkness was confirmed also by results of other studies, when spelt is used (Abdel-Aal, Hucl, Sosulski, & Bhirud, 1997; Frakolaki, et al. 2018; Kohajdová & Karovicova, 2007). Instead, the increase in crumb redness in presence of LAB might be related to a more intense Maillard reaction with consequence formation of brown compounds, following the release of amino acids content as metabolism products of LAB (Winters et al., 2019).

Replacing *S. cerevisiae* with *K. unispora* resulted in a further worsening of both the dough properties (i.e., longer leavening time and lesser CO_2 production) and the bread properties (i.e., volume and specific volume). The low amount of CO_2 produced, and the consequent limited development of bread volume, might explain the highest crumb firmness achieved by this sample (Fig. 4).

On the other hand, despite the non-conventional yeast *K. unispora* did not perform as well in fermentation, it did not affect the consumer preference when it was used alone. Whereas, when *K. unispora* was used in combination with the LAB strains, the bread was significantly less preferred (Fig. 6), probably as a result of the high crumb firmness, which is reported to be a negative contributor to bread acceptance (Laureati, Giussani & Pagliarini, 2012). Moreover, the slow leavening contributes to a higher acidity which may also lead to the lower preference observed, but the use of alternative yeasts should not be dismissed, because previous research has shown that in association with slower fermenting LAB and at lower temperatures, they may be a viable alternative for sourdough bread production at industrial level (Häggman & Salovaara, 2008). Furthermore, long fermentations in which alternative non-*Saccharomyces* yeasts, such as *K. unispora*, are used as leavening agents, seem to have a beneficial impact on bread quality (Xu et al., 2019). It should also be considered that sour taste perception varies considerably among subjects, with consumer segments accepting and preferring products characterized by higher acidity levels (Ervina, Berget & Almli, 2020; Törnwall et al., 2014).

3.6.6 Conclusions

In this study, two microbial consortia were studied for their ability to confer positive characteristics to a sourdough enriched with spelt flour, a low performant but sustainable and nutritional crop. The study highlights the importance of exploring the pro-technological features of non-conventional species, both yeasts and LAB, and their synergies, to obtain stable consortia that can be used at industrial level. Overall, the selected cultures exhibited potentials for future applications that deserve further studies to better understand the real role of these single or associated cultures in improvement of technological, nutritional, and sensory characteristics of the bakery products.

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Declaration of competing interest

No competing interest to declare.

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4. Design and application of adjunct cultures for the valorization of fine cocoa fermentation

Research conducted in the Dominican Republic at Rizek Cacao S.A.S., under the tutorship of Dr. Alberto Fanton.

4.1 STATE OF THE ART

Cocoa (*Theobroma cacao* L.) is the main ingredient of the production of chocolate. The plant is native to the Latin America, but nowadays it is cultivated in many regions of the world in the equatorial zone. The main producing countries, Ivory Coast, Ghana, Indonesia, Nigeria, Cameroon, Brazil, Ecuador, the Dominican Republic, and Malaysia make up 90% of the global cocoa productions (Aprotosoaie et al., 2015). The main original varieties are Forastero, Criollo, and Trinitario, a hybrid between the first two, although many different varieties are spread and cultivated throughout the world; in Latin America Motamayor et al. (2008) identified 10 distinct genetic clusters cultivated in the Amazon region alone.

Forastero is the most widely cultivated cocoa variety, making up 90% of the global cocoa production. This variety is characterized by high yields and high resistance to pests and climatic conditions. The beans are purple because of to the high polyphenol concentration, and the cocoa mass is characterised by strong chocolate notes.

Criollo on the other hand is the rarest cocoa variety. It is cultivated in limited areas in the Central America, Venezuela, Madagascar, Sri Lanka, and Samoa. This variety has very low productivity and is very susceptible to adverse conditions. The beans are white due to their low content in polyphenols; this gives to the cocoa mass a very low astringency and bitterness with a characteristic fine flavour, with special flowery, fruity, nutty, and earthy notes.

The Trinitario hybrid is more resistant and productive than Criollo and maintains some of the fine flavours found in this variety, with stronger chocolate notes. Its production makes up 5-10% of the total cocoa market (Aprotosoaie et al., 2015).

The demand for cocoa and chocolate is in a rising trend as an increasing number of consumers appreciate not only the unique flavour of chocolate but also the health benefits that are being recently investigated. Bulk cocoa,

mainly of the Forastero variety, is a commodity and its price is set in the stock market, on the other hand, the Criollo and Trinitario hybrids, as well as Nacional, a variety of Forastero, are classified as fine and flavour cocoa varieties, and as such, they are sold for a premium price. For this reason, the high quality of this product is essential for producers and buyers alike, as the price is determined based on specific flavour and quality parameters. Thus, every step of cocoa transformation needs to be performed at the highest standard, since nonconformities represent a major economic loss for the producers (Escobar et al., 2021).

Fermentation is one of the most crucial post-harvest transformation steps of cocoa, that gives to the product its typical notes. Indeed, unfermented cocoa is excessively bitter and astringent due to the high content in polyphenols and does not have the desirable chocolate notes of well-fermented cocoa.

For the most part cocoa fermentation is conducted in a traditional, spontaneous manner at farm level. Cocoa pulp surrounding freshly harvested cocoa beans is a good substrate for microbial fermentation. It contains 82-87% water, 10-15% fermentable sugars, mainly glucose, fructose and sucrose, 2-3% pentosans, 1-3% citric acid, 1-1.5% pectin, 1-2% hemicellulose as well as proteins, amino acids, vitamins and minerals (Puerari et al., 2012). The high sugar content and low initial pH of about 3-4 in optimal for the growth of yeasts and subsequently of LAB. Their growth is also stimulated by the low oxygen levels of the initial stages of the fermentation, condition created due to the high viscosity of the pulp that does not allow aeration. Due to the pectinolytic activity of yeasts the pulp is solubilized and drains away in the form of sweatings.

After 1-2 days of fermentation, the fermenting cocoa mass is mixed and aerated, a condition that favours the growth of acetic acid bacteria (AAB), that oxidize ethanol and sugars to produce acetic acid. This exothermic reaction significantly increases the temperature of the cocoa, from the initial 30°C to temperatures up to 50°C. The heat and acetic acid penetrate the cocoa bean and cause the death of the cotyledon. This not only prevents the germination,

but also activates the endogenous enzymes, such as the polyphenol oxidase that acts on polyphenols and reduces the astringency of cocoa, as well as proteases that liberate small peptides and free amino acids, other essential constituents of the chocolate flavour (Santander Muñoz, 2019). In these conditions the growth of LAB and yeasts is inhibited, whereas spore-forming bacilli start to grow at the late stages of the fermentation. The cocoa mass is mixed every 24-48 hours and is left to ferment generally for 4-6 days, after which it is put to dry in drying beds. The duration of the fermentation and the frequency of mixing also play a role in the quality of the final product (Camu et al., 2020).

Since the beans coming from unopened pods are considered to be microbiologically sterile, the source of the microbial species carrying out the fermentation is the environmental contamination coming from the surface of the pods, handling and transportation, residues on the surfaces of fermentation boxes, and other plant materials that may come in contact, for example, plantain leaves that often are used to cover the boxes or heaps (De Vuyst & Weckx, 2016).

The role of the different microbial groups that participate in cocoa fermentation has been studied for a long time, especially concerning the yeasts, LAB and AAB.

Yeasts are the dominant microorganisms isolated at the first stages of cocoa fermentation. Their role is essential for the outcome of the fermentation and the flavour development. The main genera of yeasts isolated from cocoa are *Saccharomyces*, *Pichia*, *Hanseniaspora* and *Candida*. The main role of yeasts is the production of ethanol and CO_2 through the alcoholic fermentation of fermentable sugars. Ethanol is the main substrate used by AAB, whereas the production of CO_2 creates favourable conditions for the growth of LAB that are more oxygen sensitive. Other metabolites produced by yeasts, such as higher alcohols, aldehydes, ketones, organic acids, esters and other volatiles penetrate the bean and thus impact the final flavour notes of chocolate.

The pectinolytic activity of yeasts is also essential to the correct aeration of the fermenting mass and thus for the development of the AAB. In fact, many yeast species isolated from cocoa were shown to be highly pectinolytic (De Vuyst & Leroy, 2020).

The role of the LAB in cocoa fermentation has not been completely understood. Initially, the presence of LAB was considered unimportant, or even detrimental to the quality of chocolate as residual lactic acid, that is less volatile than acetic acid, increases the acidity of the cocoa liquor. On the other hand, their ability to utilize citric acid is considered important for the initial increase of the pH of cocoa pulp to values closer to the optimal conditions for the enzymatic activities described previously. Also, the ability to reduce fructose to mannitol is considered relevant not only for the quality of the cocoa but possibly for the subsequent microbial communities, as this process reduces the amount of fermentable sugars available. Similarly to yeasts, LAB also produce a number of secondary metabolites, such as aldehydes, ketones, organic acids and esters that could impact the final flavour of the chocolate (Ho et al., 2015).

Since a high concentration of lactic acid is seen as detrimental to the quality of cocoa, a dominance of heterofermentative species would be preferable, as the amount of lactic acid produced is lower. Indeed, from the data available from the literature it appears that although homofermentative LAB are sometimes isolated, the dominant species are the facultatively heterofermentative *Lactiplantibacillus plantarum* and the obligately fermentative *Limosilactibacilus fermentum* (Ho et al., 2015).

Another important activity of LAB is the production of antimicrobial compounds that could influence the microbial ecology as well as inhibit the growth of toxinogenic fungi.

AAB that develop after the mixing and aeration of the fermented cocoa perform a rapid oxidation of ethanol, sugars and acids produced by yeasts and LAB or naturally present in the cocoa pulp. As mentioned, this step is crucial for the death of the cotyledon and the activation of the endogenous

enzymes. Furthermore, the high concentration of acetic acid has antimicrobial properties that not only limit the growth of yeasts and LAB but also that of contaminants such as fungi and bacilli. Since acetic acid is volatile, such concentrations are generally not problematic for the final chocolate acidity as acetic acid is removed during drying, roasting and subsequent processing of the cocoa mass. The main species of AAB isolated from cocoa is *Acetobacter pasteurianus* (Moens et al., 2014).

The presence of spore forming *Bacillus* is also reported at the late stages of cocoa fermentation. These bacteria are generally pectinolytic, proteolytic and lipolytic, so, although they may play a relevant role in combination with yeasts in the pulp degradation, their presence is generally correlated with the production of undesirable off-flavours as they produce short chain fatty acids that negatively impact the quality of chocolate.

The growth of filamentous fungi is also considered detrimental for the quality of cocoa, not only because of the production of off-flavours due to proteolytic and lipolytic activities, but most importantly for the production of mycotoxins, especially aflatoxin and ochratoxin A. Although the high fermentation temperatures greatly restrict the growth of fungi, their quantity increases during the drying phase, as such, they represent a safety and quality concern. Mycotoxin producing fungi, especially *Aspergillus* section Flavi and *Aspergillus* section Nigri have been identified in drying cocoa (Schwan et al., 2014).

Previous studies concerning the design and application of starter cultures to cocoa fermentations have mainly focused on yeasts, and especially on their pectinolytic phenotype. The scientific research is still lacking when it comes to the use of LAB and AAB as starters; most of studies where these microorganisms were included in the starter culture use the dominating species, that is, *L. plantarum* and/or *L. fermentum* for LAB and *A. pasteurianus* for AAB. For this reason, whereas variety in cocoa quality has been observed for different yeast species, it remains unclear whether the same applies to LAB and AAB (Díaz-Muñoz & De Vuyst, 2021; Pereira et al., 2016).

Although the spontaneous fermentation is still the main process used, the industry in moving towards the modernization of this process despite the fact that the complex relationships between microbial communities remain unclear. Applying a starter culture would minimize the unpredictable nature of the process minimizing the low efficiency, low qualitative consistency and safety concerns that come with the traditional fermentation process.

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4.2 AIMS AND OBJECTIVES

The main aim of this project was to perform cocoa fermentation with selected LAB as adjunct cultures and to evaluate their effect on the flavour of chocolate as well as their protective role towards mould growth. In addition, two different fermentation setups were studied to evaluate the optimal inoculation step. The objective of this chapter was to valorise fine cocoa by performing a controlled fermentation with selected LAB and to further explore their role in the fermentation process.

4.3 Exploration of *Lactiplantibacillus fabifermentans* and *Furfurilactobacillus rossiae* as potential cocoa fermentation starters

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4.3.1 Introduction

Cocoa bean fermentation is a crucial step in the production process of chocolate and its quality. This step is fundamental in determining the flavor profile and composition of chocolate, and in reducing the excessive bitterness and astringency of unfermented cocoa. At industrial level cocoa fermentation is typically a spontaneous process carried out with different methodologies such as in heaps, boxes or trays, with banana or plantain leaves sometimes used to contain the cocoa.

Cocoa beans inside the cocoa pod are sterile, and the microbial species that conduct the fermentation derive from environmental contaminations such as the surface of the pods, from the hands and machetes used to open them, from the sacks and baskets used for transportation, from fermentation boxes and plantain leaves.

The fermentation is carried out by yeasts and lactic acid bacteria (LAB) in the initial anaerobic stage. Glucose, fructose and citric acid are fermented into organic acids and ethanol by both LAB and yeasts, that also convert the pulp in simple sugars thanks to the pectinolytic activity. After 1-2 days the fermenting mass is mixed, a process that introduces oxygen and causes a shift in the microbial population: whereas the presence of yeasts and LAB decreases, aerobic acetic acid bacteria (AAB) begin to develop and convert ethanol and residual sugars into acetic acid. This phase is characterized by an increase in temperature that in synergy with the presence of acetic acid kill the embryo, activating its hydrolytic enzymes and inhibiting the germination. At the end of the fermentation spore forming *Bacillus* species and mold may grow.

Independently of the region of cultivation, the dominating LAB species in cocoa fermentation are facultatively heterofermentative *Lactiplantibacillus plantarum* and strictly heterofermentative *Limosilactibacillus fermentum* (Schwendimann et al., 2015). Other species such as *Fructobacillus* spp. and *Leuconostoc* spp. have been reported to play an important role at the beginning of the fermentation, however, previous starter culture design

experiments for cocoa fermentation have focused on the former two species (De Vuyst & Weckx, 2016).

The goal of this work was to evaluate the potential of two autochthonous strains of LAB belonging to the species *Lactiplantibacillus fabifermentans* and *Furfurilactibacillus rossiae*, isolated from fermented cocoa, to be used as adjunct cultures for an improved cocoa bean fermentation process.

These species are not considered to be dominant in cocoa fermentations. Indeed, the facultatively heterofermentative *L. fabifermentans* first described by De Bruyne et al. (2009) from Ghanaian cocoa fermentations, was reported as a minority species at the first stages of Ecuadorian cocoa fermentation (Papalexandratou et al., 2011), and represented only 1.23% of the LAB isolated from fermented cocoa in the Côte d'ivoire (Adiko et al., 2018), although, because of the high similarity with *L. plantarum*, this species may have been underestimated in previous cocoa fermentation microbiota research. *L. fabifermentans* has one of the biggest genomes of LAB which shows a great genomic versatility, with a wide range of carbohydrate utilisation (Campanaro et al., 2014). The reported preference for fructose, rather than glucose, can reduce the competition with yeast, making this species a good candidate for a mixed starter culture (Lefeber et al., 2011).

F. rossiae, on the other hand, has never been described in cocoa fermentations before, to the authors' knowledge. This obligately-heterofermentative LAB has been isolated from a wide range of environmental niches, such as sourdoughs, fruit, fermented meat, and animal and human gut. This species can metabolise a substantial number of carbohydrates, and genomic research has shown its potential for polysaccharide degradation, as revealed by an in-silico analysis (De Angelis et al., 2014).

This research aims to investigate some characteristics of these minority, scarcely studied species, with particular regard to those properties that could be exploited in an improved cocoa fermentation process from a quality and safety point of view.

4.3.2 Materials and methods

4.3.2.1 Strain isolation and maintenance

Microbial population was isolated from a “light-breaking” cocoa variety provided by Rizek Cacao S.A.S., San Francisco de Macorís, Dominican Republic, at different fermentation times. The LAB isolation was performed by a culture-dependent method, plating adequate dilutions in Man Rogosa Sharpe (MRS) agar plates. For each fermentation day, approximately 10 colonies were isolated. Pure cultures of each strain were routinely subcultured in MRS broth (Difco Lab., Augsburg, Germany) at 30°C for 24h.

Similarly, yeast strains were isolated by plating adequate dilutions in Yeast extract Glucose Chloramphenicol (YGC) agar plates (MilliporeSigma, St. Louis, Missouri, US). Isolated strains were maintained in Yeast extract Peptone Dextrose (YPD) broth incubated at 30°C for 24h. The composition of the YPD medium is (g L^{-1}): yeast extract 10, glucose 20, peptone 20 (MilliporeSigma, St. Louis, Missouri, US).

Mold isolates were obtained from samples of cocoa beans at the end of the fermentation period. Mold strains belonging to *Aspergillus flavus, Aspergillus niger, Mucor circinelloides and Fusarium verticillioides* were also used in this study. Molds were cultured in Malt Extract Agar (MEA) plates (Thermo Fisher Scientific, Massachusetts, US). Spores were collected by pouring sterile distilled water containing 0.9% NaCl on the plates after complete sporification, slowly agitating and storing in sterile tubes.

All isolates were deposited in the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy, at −80° C in their maintenance medium, with the addition of 15% glycerol.

L. fabifermentans SAF13, *F. rossiae* SAF51, *L. plantarum* B7 and *Saccharomyces cerevisiae* TB2.3 were selected for further studies.

Acetobacter pasteurianus DSM 3509 was used for co-culture experiments. The strain was maintained in Glucose Yeast extract Calcium Carbonate (GYC) agar plates incubated at 28°C for 48h. The composition of the GYC

agar medium is as follows (g L^{-1}): glucose 50, yeast extract 10, calcium carbonate 30, agar 15 (MilliporeSigma, St. Louis, Missouri, US).

4.3.2.2 Molecular identification of the isolates and q-PCR experiments

The identification of yeast isolates was performed by 26S rRNA gene sequencing or ITS amplification and restriction with restriction enzymes *Hind*III and *Hin*fI, as previously reported (Decimo et al., 2017). Mold identification was carried out by 26S rRNA gene sequencing.

LAB isolates were identified by 16S rRNA gene sequencing. For the subsequent detection of *F. rossiae* and *L. fabifermentans* both in single culture and in co-cultures, species-specific probes were used or designed. For the detection of *F. rossiae*, primers designed by Da Riedl et al. (2017) were used. For the detection of *L. fabifermentans*, primers were designed in this study using the 16S rRNA gene deposited in the GeneBank database and aligning to the 16S rRNA genes of the affine *L. plantarum* species to check for specificity. Furthermore, the melting temperature (TM) was optimised to avoid mismatches. The primers and thermal cycles are reported in Table 1. For qPCR experiments, calibration curves were designed by inoculating decimal dilutions of an overnight grown culture of each *F. rossiae* and *L. fabifermentans* strain in a range from 3-8 log cycles in MRS broth. Cell lysis of the bacterial pellet recovered from MRS and DNA extraction was performed as reported in Mora et al. (2000).

The PCR reaction was carried out in a total volume of 15 µL, containing 7.5 µL of qPCR mix (SSO Fast Supermix, BioRad, Hercules, USA), 0.36 µL of each primer (0.3 µM), 1.78 µL of PCR grade water and 5 µL of DNA. The efficiency of the standard curves was in the range 90-110% and the R^2 was >0.99.

Tab.1. Primers and thermal cycles for species-specific PCR analyses

Species	Primer sequences	Thermal cycle	Reference
L. fabifermentans	F: CTGGTATTGATTGGTACTTGT R: ACCTCACCATCTAGCTAATG	95 °C × 10 s 59 °C × 20 s 72 °C × 20 s	This study.
F. rossiae	F: GGCGTGCCTAATACATGCAA R: TGTCTCGTCAATCTGGTGCAA	95 °C × 10 s 60 °C × 20 s 72 °C × 20 s	Riedl et.al, 2017.

4.3.2.3 Tolerance to stress conditions and metabolic characterisation

The ability of *L. fabifermentans* SAF13 and *F. rossiae* SAF51 to resist to different stress conditions were tested in MRS broth at different temperatures (from to 25 to 42°C) and pH values (from 3.0 to 6.5). The tolerance to high osmolarity was evaluated in MRS broth containing 15% and 30% glucose or fructose. The growth was evaluated by measuring the Optical Density at 600nm (OD_{600nm}). The autochthonous *L. plantarum* strain B7 was used as comparison. The survival rate was measured as $(OD_C-OD_T)/OD_C$ where OD_C is the OD_{600nm} at standard conditions and OD_T is the OD_{600nm} at the test conditions.

The growth of the strains in presence of glucose, fructose and sucrose was tested in MRS broth containing 1% of each sugar. Overnight grown cultures of each strain were twice washed with sterile saline water and inoculated in 200µL of medium at a concentration of 10^6 CFU mL^{-1} in 96 well plate. The growth was measured using an automated microplate reader (Eon™ Microplate Spectrophotometer, BioTek, Vermont, US) at 600nm. The lag time was calculated by the instrument.

The production of acetic acid and lactic acid after 24h incubation at 30°C was measured using commercial assay kits according to the manufacturer's instructions (R-Biopharm AG, Darmstadt, Germany).

4.3.2.4 Autolytic activity

The autolytic property of the strains was determined according to the method described by Ayad et al. (2004). The cell pellets were resuspended in citrate buffer (0.1M, pH 5.5) containing 0.5 M NaCl and then diluted to $OD_{650\ nm}$ =1.0. The suspension was incubated at 30°C for 48h.The rate of autolysis was determined after reading the OD_{650nm} using the formula %autolysis = (1-OD_{48h} /OD_{T0}) *100.

4.3.2.5 Peptidase activity

To investigate the peptidase system of the strains, a cellular pellet was recovered from an overnight grown culture by centrifuging at 8000g for 10 min at 4°C. The pellet was twice washed and resuspended in sterile saline solution (0.7% NaCl) and the cells were ruptured in a TissueLyzer (Qiagen, Hilden, Germany) at $30s^{-1}$ for 6 min. The peptidase activity was evaluated in the cell free extracts using paranitroanilide (pNA) substrates, according to Iametti et al. (2002). In addition, N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BApNA) and N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SUpNA) were used as substrates to assess the proteolytic activity. The reactions were carried out in a 0.05M sodium acetate buffer at pH 4.5. The hydrolysis of the substrate was evaluated by measuring the OD at 405nm and the results are expressed as increment of absorbance (mOD) per hour per mg of protein. The protein content of the cell free extracts was evaluated according to Bradford, 1976.

4.3.2.6 Antifungal activity assay

Antifungal activity was tested using the overlay method (Axel et al., 2016). Each LAB strain tested was streak plated in MRS agar and grown for 72h at 30°C. Afterwards a layer of MEA soft agar (0.7% agar) containing 10^4 /mL spores suspension was slowly added to the plates that were incubated for 3 days at 30°C. Antifungal activity was evaluated as clear zones of inhibition around the bacterial smears.

4.3.2.7 Evaluation of growth in cocoa

The bacterial growth ability was evaluated in cocoa pulp. For this purpose, cocoa pods were harvested, and the surface was cleaned with alcohol. The pulp was collected in sterile conditions and was pasteurised (65°C for 30 min), to ensure the absence of contamination. The sterility was confirmed by plating in MRS agar, YPD agar and PCA and incubating at 30°C for 48h. Approximately 10^5 CFU/mL of each LAB strain tested were inoculated in the cocoa pulp and the growth was evaluated by dilution and plating in MRS agar after 16 and 40h of incubation.

4.3.2.8 Growth in co-culture

To investigate the possibility of *F. rossiae* and *L. fabifermentans* strains to be used in conjunction with one another, co-culture assays were set up. The growth was performed in MRS broth by inoculating 10^5-10^6 CFU/mL of each strain and incubating at 30°C for 16 and 40h. The growth of each strain was evaluated by qPCR.

The two LAB strains were also grown in conjunction with the autochthonous strain *S. cerevisiae* TB2.3. The growth of the yeast was evaluated with the plate count method in YGC agar and the growth of bacterial strains was evaluated by qPCR assay.

Co-cultures of LAB strains and *A. pasteurianus* were carried out by inoculating 10^6 CFU/mL of cells in MRS broth in agitation at 150 rpm and 28°C. The growth of LAB was evaluated by plate count in MRS agar incubated in anaerobic conditions, the growth of *A. pasteurianus* was evaluated in basal MRS agar medium without glucose and with 4% ethanol as sole carbon source.

4.3.2.9 Statistical analysis

All results are expressed as mean ± SD of three independent replicates of each experiment. Unpaired two-tailed t-tests were performed to assess for statistical significance using GraphPad Prism 8 (v. 8.4.3, GraphPad Software

Inc., California, USA). Differences were considered statistically significant for $p<0.05$.

4.3.3 Results

4.3.3.1 Microbial population composition

The LAB and yeast populations isolated from the Dominican cocoa fermentation are reported in Table 2. The dominating LAB species was *L. plantarum*, that was present throughout all days of fermentation. Apart from other species commonly associated with fermented cocoa, such as *L. paracasei* and *L. brevis,* minority species such as *L. rhamnosus*, *F. rossiae*, *L. fabifermentans* and *L. satsumensis* were also isolated.

The LAB population had a larger diversity in the first 48h of fermentation. After 72h, in correspondence with the mixing of the cocoa mass, the biodiversity of LAB was significantly reduced, and the population was comprised of mainly *L. plantarum*.

The dominating yeast species was *S. cerevisiae*, which is also typical of cocoa fermentations. This species represented 65% of all isolates identified. Other identified yeasts, such as *Hanseniaspora opuntiae*, *Torulaspora delbrueckii* and *Pichia* spp., are also typical of fermented cocoa.

The four mold strains isolated from moldy cocoa samples belonged to the species *Aspergillus tamarii, Aspergillus nidulans, Lichtheimia ornata* and *Rhizomucor pusillus.* These species occur commonly in tropical and subtropical regions; their presence during cocoa fermentation, especially in the last days of fermentation, has been documented in different geographical areas (Copetti et al. 2014; Delgado-Ospina et al. 2021).

4.3.3.2 Carbon source utilization, lactic and acetic acid production and tolerance to stress conditions

L. fabifermentans and *L. plantarum* utilized glucose, fructose and sucrose as carbon sources, whereas *F. rossiae* could only use glucose and fructose. Moreover, *F. rossiae* had a significantly higher lag time (14.3±0.01 h) compared to *L. plantarum* (4.6±0.006 h) and to *L. fabifermentans* (3.7±0.01 h), when grown either in glucose or in fructose.

Tab. 2. LAB and yeast species isolated at different fermentation times.

* Number of isolates

Microbial species	Fermentation time (h)					Total isolates	Percentage
	24	48	72	96	120		
LAB species							
L. plantarum	0	9*	2	23	10	44	73.33
L. paracasei	0	2	0	2	0	4	6.67
L. brevis	0	1	2	0	0	3	5
L. rhamnosus	0	1	0	0	2	3	5
L. fabifermentans	0	0	3	0	0	3	5
F. rossiae	0	2	0	0	0	2	3.33
L. satsumensis	0	0	0	1	0	1	1.67
Yeast species							
Saccharomyces cerevisiae	7	14	16	9	7	53	65.43
Torulaspora delbrueckii	3	0	0	4	0	7	8.64
Schizosaccharomyces pombe	0	0	0	0	6	6	7.41
Hanseniaspora opuntiae	3	2	0	0	0	5	6.17
Wickherhamomyces pijperi	3	0	0	0	0	3	3.7
Starmerella bacillaris	1	2	0	0	0	3	3.7
Pichia kudriavzevii	2	0	0	0	0	2	2.47
Pichia manshurica	0	2	0	0	0	2	2.47

After 24 h of growth, *L. fabifermentans* and *L. plantarum* produced mainly D-L lactic acid: its final concentration (about 11 g L^{-1} L-lactic acid and 7.5 and 6.3 g L^{-1} D-lactic acid for *L. fabifermentans* and *L. plantarum* respectively) was 53 times higher than the concentration of acetic acid for both strains (0.33-0.34 g L^{-1}). *F. rossiae* also produced lactic acid as the main product of its fermentation, but the proportion of acetic acid was closer to 1:2 (3.71 g L^{-1} D-lactic acid, 4.91 g L^{-1} L-lactic acid and 3.5 g L^{-1} acetic acid).

All tested strains showed a good resistance to cocoa-related stress conditions such as low pH, high temperature and high osmotic pressure (Fig.1). *L. fabifermentans* and *L. plantarum* had an optimal growth at pH 6, but a moderate level of growth was also observed at pH 3; *F. rossiae* was more sensitive to low pH values, showing very limited growth at pH 3. *L. fabifermentans* was the most resistant strain to high temperatures, growing at temperatures up to 42°C. No growth was registered at 50°C.

4.3.3.3 Autolysis

The autolytic phenotype of the LAB species under investigation was studied at pH 4 and 0.5M NaCl to simulate some of the conditions of cocoa pulp. The results indicate that *F. rossiae* SAF51 and *L. plantarum* B7 have a moderate autolysis rate, as the results obtained were 28% and 23.9%, respectively. *L. fabifermentans* SAF13 on the other hand had a very limited autolytic ability, as the OD was reduced by only 5.4%.

4.3.3.4 Antifungal activity

The ability of the two selected strains to inhibit the fungal growth was evaluated in comparison with the strain *L. plantarum* B7.

As shown in Fig. 2, *L. fabifermentans* and *L. plantarum* showed the highest inhibitory activity towards the mold species that can be found at the final stages of cocoa fermentation. Particularly, *L. fabifermentans* was the most effective strain in inhibiting *L. ornata* and *A. tamarii*. *F. rossiae* showed low level inhibition towards *R. pusillus* and no inhibition towards *L. ornata* and *A. tamarii*, whereas it could efficiently inhibit the growth of *A. nidulans*. Moreover, the three LAB tested showed interesting antifungal activity toward *A. flavus* and *F. verticillioides*, while no inhibition was evaluable for *A. niger* and *M. circinelloides.*

Fig. 1 Growth at different pH (A) and temperatures (B) and survival rate relative to standard MRS medium (C) of the strains *F. rossiae* SAF51 (■), *L. fabifermentans* SAF13 (■) and *L. plantarum* B7 (■).

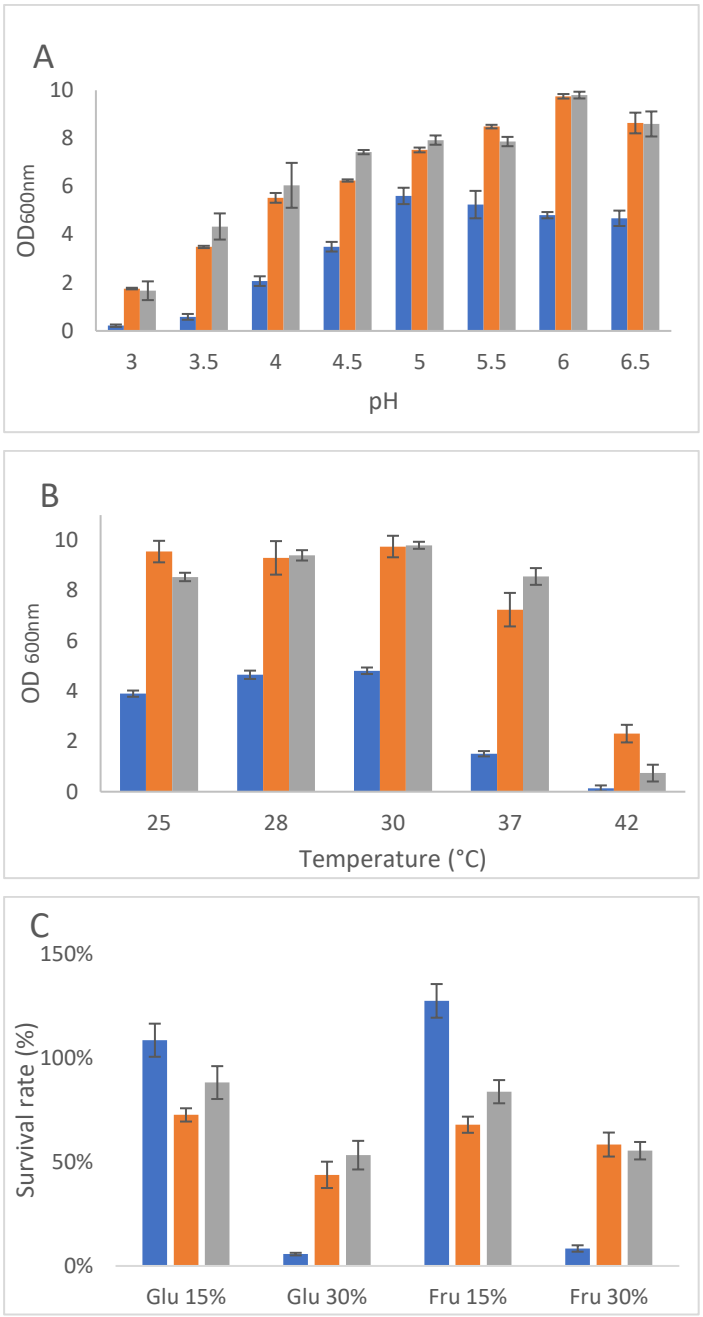
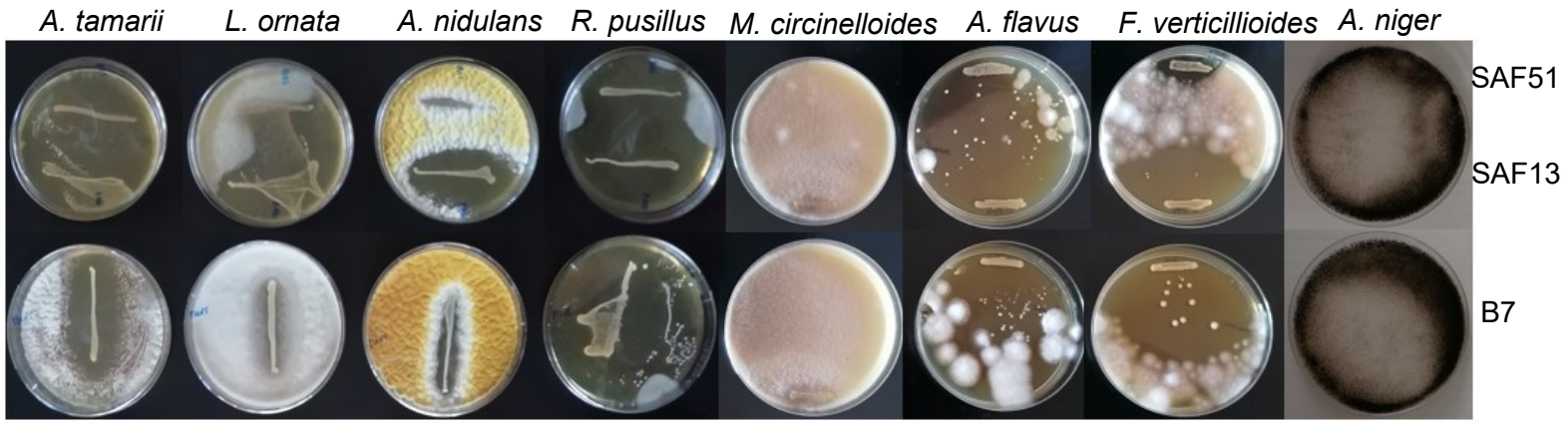


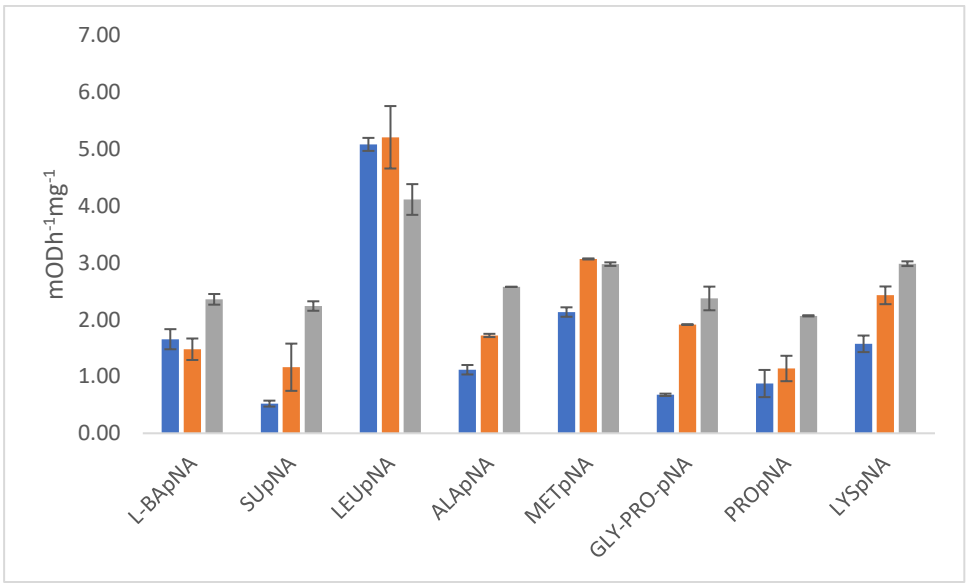
Fig. 2. Antifungal activity overlay assay of strains *F. rossiae* SAF51, *L. fabifermentans* SAF13 and *L. plantarum* B7. Antifungal activity is represented by the clear zone surrounding the bacterial smear.



4.3.3.5 Peptidase activity

Peptidase activities of the strains *L. fabifermentans* SAF13, *F. rossiae* SAF51 and *L. plantarum* B7 are shown in Fig. 3. All strains possessed a complex pool of peptidases such as aminopeptidases that were especially active on hydrophobic aminoacids such as leucine, with a significant activity on alanine, methionine and lysine, iminopeptidases that hydrolyse proline residues and prolylpeptidases, active on Gly-Pro-pNA. Furthermore, proteolytic activity was also observed. *L. fabifermentans* SAF13 had a significantly higher activity on all tested substrates except for LEU-pNA and MET-pNA, whereas *F. rossiae* SAF51 in general was the strain that possessed the lowest peptidasic activity.

Fig. 3. Peptidasic activity of strains *F. rossiae* SAF51 (■), *L. fabifermentans* SAF13 (■) and *L. plantarum* B7 (■) expressed in mOD h^{-1} mg^{-1}.



4.3.3.6 Growth in cocoa and in co-culture

The growth of *L. fabifermentans* and *F. rossiae* strains in cocoa pulp is represented in Fig. 4. Compared to the growth in liquid MRS broth, the growth in cocoa pulp is slower, but after 40h the final cell concentration in the two conditions is similar.

Mixed culture experiments were set up in MRS broth (Fig. 5). Firstly, the ability of each LAB strain to grow in association with the cocoa related yeast strain *S. cerevisiae* TB2.3 was investigated. After 16h or 40h of incubation, the yeast growth was slightly limited by the presence of the LAB strains. This could indicate a competition for nutrients, rather than an inhibition of the yeast growth by the two LAB strains.

In the three-strain combination, whereas the growth of *L. fabifermentans* SAF13 and *S. cerevisiae* TB2.3 closely resembled the growth in two-strain co-cultures, *F. rossiae* SAF51 was strongly inhibited. Under these conditions *L. fabifermentans* takes over *F. rossiae*, presumably in relation to the higher lag time observed for the latter. Another possible cause, which deserves to be deepened, is the ability of *L. fabifermentans* to produce bacteriocin-like proteins, capable of inhibiting or delaying the growth of *L. rossiae*. In fact, Campanaro et al. (2014) previously observed that the sequenced genomes of *L. fabifermentans* present in the NCBI database contain a series of genes related to bacteriocin production and bacteriocin resistance.

When grown in association with the strain *A. pasteurianus* DSM 3509, *F. rossiae* SAF51 increased of about 4 log cycles its growth and had no effect on growth of the *A. pasteurianus* strain. The same results were observed when *L. fabifermentans* SAF13 was grown in association with *A. pasteurianus*. In this case the growth of *A. pasteurianus* incremented of about 1 log cycle, in relation to the presence of the strain *L. fabifermentans* SAF13.

Fig. 4. Growth in MRS (empty shapes) and cocoa pulp (full shapes) of the LAB strains. ■ *L. fabifermentans* SAF13 ▲ *F. rossiae* SAF51.

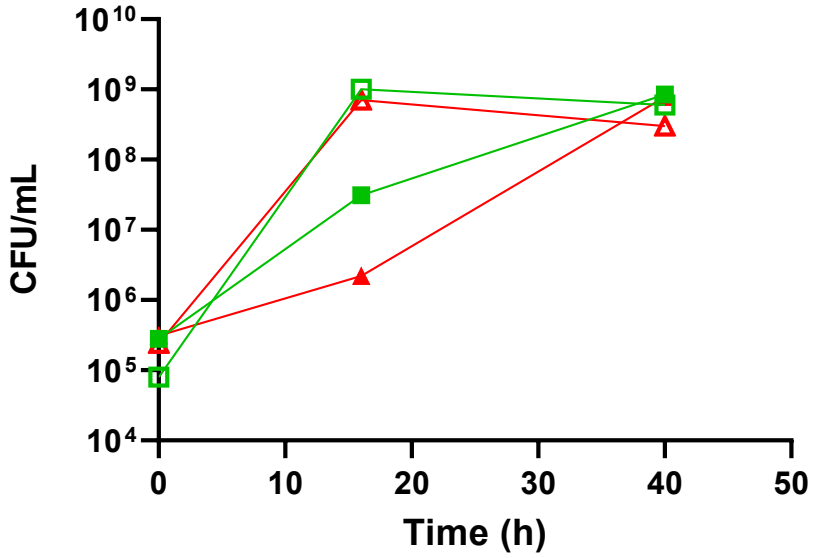
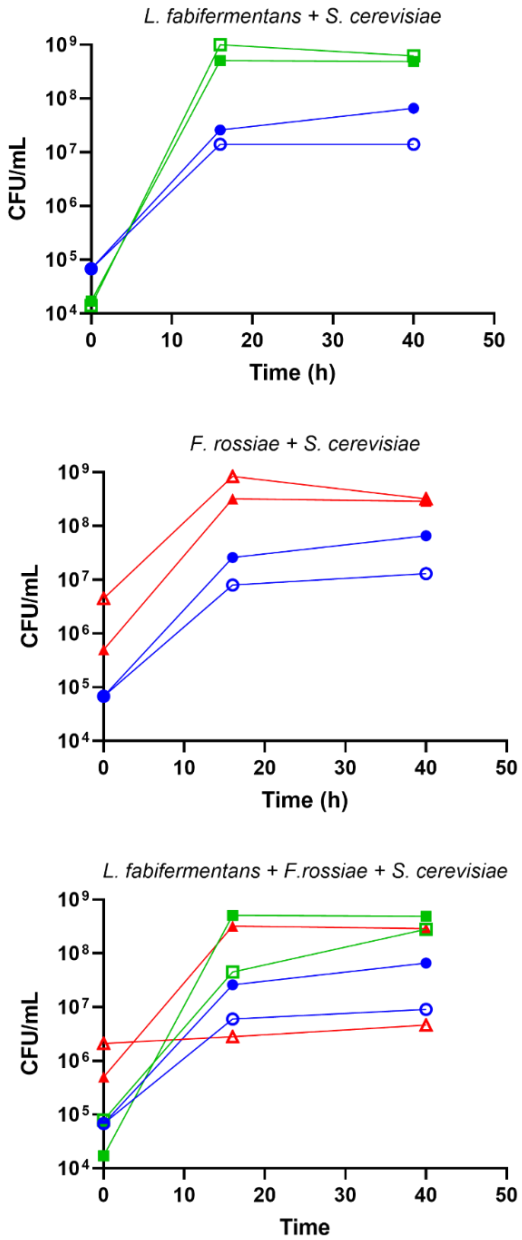


Fig 5. Viability of strains grown in monoculture (full symbols), or co-culture (empty symbols) grown in MRS broth and analysed at 16 and 40h. The two LAB strains in the three-strain mixed culture were discriminated by qPCR. ■ *L. fabifermentans* SAF13 ● *S. cerevisiae* TB 2.3 ▲ *F. rossiae* SAF51. Standard deviations are omitted for clarity.



4.3.4 Discussion

The variable nature of fermented cocoa is to this day a major problem for the cocoa and chocolate producing industries. With all other factors remaining unchanged, the outcome of the spontaneous fermentation is still difficult to control, and this is a drawback both for chocolate makers that need to use cocoa blends to maintain the uniformity of their recipes, and for cocoa producing companies for which the variable quality is often translated in an economic loss. Although the understanding of the cocoa fermentation process and the setup of controlled fermentations have been an important step towards a more controlled process, a wider understanding of the impact of the different bacterial species on the fermentation is needed to design cultures that have a positive role in the flavour and quality of cocoa.

In this research we isolated LAB and yeast strains from spontaneously fermented cocoa, from which we selected the strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 whose potential role in fermentation was investigated. In order to add to the body of knowledge on these poorly characterized species, we studied phenotypical attributes of interest, such as the ability of these strains to grow at low pH, high temperatures and high osmotic pressure, as the most important stressors of fermenting cocoa. In comparison to a cocoa related strain of the most representative species, *L. plantarum* B7, *L. fabifermentans* SAF13 behaved in a similar fashion in these conditions, demonstrating a good ability to survive and grow at low pH and high temperature, up to 42°C. *F. rossiae* SAF51, on the other hand, grew less efficiently in all conditions except in 15% of sugar added medium, the approximate sugar content of cocoa pulp (Schwan & Wheals, 2004), in which this strain not only performed better than the other two strains, but grew more efficiently than in standard MRS medium (1% glucose). Furthermore, the optimal pH for the growth of this strain was 5, lower than the strains of *L. plantarum* and *L. fabifermentans* that grew better at pH 6. This demonstrates the good adaptation that the strain of *F. rossiae* has for conditions like those

of fermenting cocoa: indeed, in cocoa pulp its growth after 40h was comparable to *L. fabifermentans* and to the growth in standard MRS medium. The two strains behaved differently during the fermentation of glucose, fructose and sucrose, the main fermentable sugars present in cocoa pulp (Afoakwa et al., 2013). *L. fabifermentans*, similarly to *L. plantarum*, grew efficiently using all three substrates, whereas *F. rossiae* had significantly longer lag times when growing in glucose and fructose, and as reported on Corsetti et al. (2005) the species cannot ferment sucrose. The inability ferment sucrose makes this species adequate for co-culture growth as it reduces the competition for the carbon source.

Despite some beneficial activities have been proposed, generally the growth of mold in cocoa is considered non beneficial for the quality of the chocolate. Their growth during the pre-processing stages of chocolate production, mainly during drying and storage, poses a significant risk due to the production of mycotoxins, mainly ochratoxin A and aflatoxin (Copetti et al., 2013). Given the stable nature of these compounds, prevention becomes key to avoiding their presence in the production chain. The ability of the strains of *F. rossiae*, and especially *L. fabifermentans* to inhibit the growth of aflatoxinogenic species *A. flavus*, as well as other toxin-producing *Aspergillus* species isolated from cocoa makes these strains good candidates for the biocontrol of aflatoxin-producing mold species. From the literature, strains belonging to *A. tamarii* have been indicated to being producers of aflatoxins (Klich et al., 2000), whereas *A. nidulans* produces sterigmatocystin, a toxic precursor of aflatoxins (Keller & Adams, 1995).

In general, the two strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 showed a high level of adaptation to the cocoa matrix, had a good resistance to stress factors related to the fermentation and possessed interesting activities that can be beneficial in a controlled cocoa fermentation process.

4.3.5 References

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4.4 Fine cocoa fermentation with selected Lactic Acid Bacteria: fermentation performance and impact on chocolate flavour

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4.4.1 Introduction

Cocoa (*Theobroma cacao L*.) is the main ingredient in the production of chocolate, one of the most important luxury foods with a rapidly expanding market. Before being suitable for chocolate production, cocoa seeds extracted from cocoa pods need to undergo a series of transformations, such as fermentation, drying and roasting. Each of these steps play an important role in the development of the typical chocolate flavour and in the reduction of undesirable notes such as bitterness and astringency (De Vuyst & Weckx, 2016).

From a quality standpoint, the cocoa beans are classified into “fine” or “flavour” cocoa and “bulk” cocoa. Generally, fine cocoa is produced from Criollo, Trinitario and Nacional cocoa varieties, whereas bulk cocoa is obtained from Forastero varieties. According to the International Cocoa Agreement (2010), fine cocoa can be defined as “cocoa that is recognised for its unique flavour and colour”. Although fine and flavour cocoa makes up a small percentage of the cocoa market, estimated around 12%, the demand for this product has increased rapidly in recent years. Only a restricted number of countries can export fine cocoa, and Ecuador, the Dominican Republic and Peru are the main exporters of this product (ICCO, 2021).

In order to be classified as fine cocoa, the product has to meet a number of criteria from a qualitative point of view, but the main factor is the flavour. In specific, fine cocoa has fewer undesirable flavours such as acidity, bitterness and off-flavours, and more fine flavours such as fruity, flowery, nutty, herbal or caramel notes. Obtaining these flavour profiles in a consistent manner can be problematic at industrial level where cocoa fermentation is still carried out in a spontaneous, in-farm process (Kadow et al., 2013). For this reason, the use of specific starter or adjunct cultures has been previously investigated (Crafack et al., 2013; de C. Lima et al., 2021; Lefeber et al., 2012; Magalhães da Veiga Moreira et al., 2017; Pereira et al., 2012) with the aim of standardizing the fermentation process.

The aim of this work is to evaluate the impact of adjunct cultures of selected Lactic Acid Bacteria (LAB) on fermentation parameters and flavour profile of fine cocoa and chocolate. Two strains of LAB, *Lactiplantibacillus fabifermentans* SAF13 and *Furfurilactibacillus rossiae* SAF51, previously isolated from spontaneously fermented cocoa and investigated for their potential as starter or adjunct cultures (Korcari et al., 2021), were studied for their ability to modify the flavour profile of a fine, “light-breaking” cocoa variety.

4.4.2 Materials and methods

4.4.2.1 Strain growth and maintenance

Two LAB strains, *L. fabifermentans* SAF13 and *L. rossiae* SAF51 previously isolated from spontaneously fermented cocoa were cultivated in Man Rogosa Sharpe (MRS) broth (Difco Lab., Augsburg, Germany) at 30°C for 24h. The strains were maintained in MRS with 15% glycerol at -80°C and were deposited at the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy.

For the inoculation, strains grown as reported above were centrifuged at 5000 rpm for 20 min at 4°C and the cellular pellet was resuspended in a 5% sucrose solution to a final cell concentration between 10^8 and 10^9 CFU/mL. The vitality of the suspension was evaluated by plate counting in MRS agar incubated at 30°C for 48h.

Four mold strains previously isolated from cocoa and deposited at the culture Collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy, were used for the biocontrol assay. The mold strains were cultured in Malt Extract Agar (MEA) plates (Thermo Fisher Scientific, Massachusetts, US). Spores were collected by pouring sterile distilled water containing 0.9% NaCl on the plates after complete sporification, slowly agitating and storing in sterile tubes.

4.4.2.2 Fermentation setup

The cell suspension obtained was used to inoculate 60 kg of freshly harvested cocoa beans of the Criollo variety (clones IML-9, 11, 16 and 79), provided by Rizek Cacao S.A.S., San Francisco de Macorìs, Dominican Republic. The final cell concentration for each strain was between 10^6 and 10^7 CFU/g. The fermentation was carried out in closed plastic containers, that were perforated to allow an adequate pulp drainage. The fermenting mass was mixed every 48h for a total of 6 days of fermentation.

Two different inoculation setups were performed: in the first setup, the inoculation was carried out at the beginning of the fermentation, whereas in

the second setup the inoculation was performed both at the beginning and after 48h. A mixed culture fermentation comprised of both strains was also set up to explore the synergies between the strains. Finally, a control fermentation without inoculation was also performed (Tab. 1).

Tab. 1 Inoculation protocols and microbial counts for each inoculum.

Sample	Fermentation protocol	Inoculum (CFU/g of cocoa)
Control	Spontaneous fermentation	-
A	Inoculated with strain *L. fabifermentans* SAF13 at t0 only	$6.25*10^6$
B	Inoculated with strain *L. fabifermentans* SAF13 at t0 and t48	$6.25*10^6$ at t0 $1.6*10^7$ at t48
C	Inoculated with strain *F. rossiae* SAF51 at t0 only	$1.4*10^7$
D	Inoculated with strain *F. rossiae* SAF51 at t0 and t48	$1.4*10^7$ at t0 $1.9*10^7$ at t48
E	Inoculated with both strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 at t0 and t48	$6.25*10^6$ SAF13 + $1.4*10^7$ SAF51 at t0 $1.6*10^7$ SAF13 + $1.9*10^7$ SAF51 at t48

4.4.2.3 Fermentation parameters measurement

For each trial, total LAB and yeast counts were performed in MRS agar with 0.1% cycloheximide (Merck, NJ, US) and Sabouraud Dextrose Agar with 0.1% Tetracycline (Merck, NJ, US), respectively. The temperature was recorded daily. The pH of the pulp was evaluated by resuspending 10g of cocoa in 90mL of distilled water. Similarly, the cotyledon pH was measured by resuspending 10g of pulp-free cocoa beans in 90mL of distilled water.

4.4.2.4 Free amino acids profile

For the extraction of free amino acids, 4.5g of finely grinded dry cocoa beans were dispersed under magnetic stirring in 40 mL of a sodium citrate buffer solution (0.2N, pH 2.2) for 40 min. The solution obtained was placed in an ultrasound bath for 5 min and filtered in Whatman No. 41 filters (Whatman plc, Maidstone, UK). 10 mL of the filtrate were deproteinated with 10 mL solution of 7.5% (p/v) sulfosalicylic acid at pH 1.75, under magnetic stirring, for 5 min. After the addition of 250µL of Nor-Leucine as internal standard the solution was brought at a final volume of 25mL that was filtered using a Whatman No. 42 filter (Whatman plc, Maidstone, UK) and subsequently it was syringe filtered using a 0.2µm size pore syringe filter.

100µL of the suspension obtained were used for quantification by Ion Exchange Chromatography (IEC) using a Biochrom 30+ chromatograph equipped with an automatic sampler, as described by Hogenboom et al. (2017). An unfermented dry cocoa sample was used to evaluate the changes in the concentration of free amino acids due to the fermentation process.

4.4.2.5 Volatile molecules assay

To analyse the aromatic compounds of the cocoa liquor, 100mg ± 3 mg of sample were inserted in 20 mL headspace vials. Each sample was concentrated in a Gerstel MPS 2 with Dynamic Headspace DHS instrument (GERSTEL GmbH & Co., GERSTEL Inc. USA) at 70°C under 750 rpm agitation. The volatile fraction was trapped with helium (10 min, 20°C), and analysed in GC-MS (Agilent GC 6890N, Agilent Technologies Inc. USA) equipped with an Agilent DB 624 column (60 m x 0,32 mm x 1,80 µm) after thermodesorption (heatrate 30-260°C). The carrier gas was helium at a flow rate of 2mL/min. The oven temperature was set at a heatrate of 40-255°C. The mass detector was an Agilent MSD 5975 with an electronic ionisation at 70 eV. The data was analysed using the Agilent ChemStation software.

4.4.2.6 Sensory analysis

Chocolate samples for sensory evaluation were prepared at the KahKow Experience, Santo Domingo, Dominican Republic. The formulation used was cocoa liquor 58%, deodorized cocoa butter 10%, powdered sugar 32%. The chocolate was stored at a temperature between 12-20°C until the sensory evaluation.

In order to describe dark chocolate samples sensory properties, the sensory profile method was applied (ISO 13299, 2010).

Ten subjects (7 women and 3 men aged between 20 and 33 years) were selected from a panelist pool consisting of University of Milan students and employees. To participate in the study, subjects had to like and habitually consume dark chocolate.

The method consisted of a first training phase to acquire familiarity with the product and the methodology, followed by a second phase focused on the sample evaluation. Subjects were trained over a period of one month (nine sessions of approximately 1 h each). During this phase, several commercial dark chocolates were selected and presented to the assessors in order to provide a wide range of sensory variability for each attribute and thus stimulate the generation of descriptors. As training progressed, descriptive terms and relevant reference standards were defined through panel discussion.

Fourteen sensory descriptors covering appearance (brown color), odor (cocoa, red fruits, toasted, honey), taste (sweet, bitter, sour), flavor (cocoa, red fruits, toasted, honey) and texture (hardness, astringency) were defined (see Table S1 supplementary materials).

Once the vocabulary was set up, the assessors performed three preliminary sessions to acquire familiarity with the scale.

After the training phase, judges evaluated the six chocolate samples (Control, A, B, C, D, E) in three replicates. Samples A and B were inoculated with *L. fabifermentans* at t0 and t0+t48 respectively, samples C and D were inoculated with *F. rossiae* at t0 and t48, respectively and sample E was inoculated with the consortia *L. fabifermentans* and *F. rossiae* at t0 and t48.

The control was fermented without inoculation. The three replicates were performed in different days at the sensory laboratory of the Department of Food, Environmental and Nutritional Sciences (DeFENS, University of Milan) designed according to ISO guidelines (ISO 8589, 2007). Assessors were asked not to smoke, eat or drink anything, except water, for 1 hour before the tasting sessions.

Assessors received one piece of each sample, for a total of six chocolates in each tasting session and rated the intensity of each sensory attribute using a 9-point scale ("1" = "absence/minimum intensity of the descriptor"; "9" = "highest intensity of the descriptor"). The judges were instructed to assess the appearance, smell and then taste each sample.

Chocolate samples were stored and served at room temperature with plastic plates that were coded with 3-digit numbers. The order in which the samples were presented was systematically varied over assessors and replicates in order to balance the effects of serving order and carryover (Macfie, Bratchell, Greenhoff, & Vallis, 1989). Chocolate samples were evaluated in individual booths under white light. The assessors were provided with unsalted crackers and water to clean their mouths during the evaluation. The data were collected using Fizz software v2.47 (Biosystemes, Couternon, France).

4.4.2.7 Mold growth inhibition

To assess the ability of the inoculated strains to prevent mold growth, 20 g cocoa samples were retrieved at the end of the 6-day fermentation period and were inserted in sterile 50mL tubes. A 0.5 mL suspension of approximately 10^4 fungal spores of four mold species isolated from cocoa belonging to *Aspergillus tamarii*, *Aspergillus nidulans*, *Rhizomucor pusillus* and *Lichteimia ornata* was added to each tube, that were incubated at 30°C for 3 days. A non-inoculated sample was also incubated as a control. The growth of mold was evaluated visually.

4.4.2.8 Statistical analysis

Experiments reported were performed in triplicate, and results are shown as mean ± standard deviation. T-tests were performed using GraphPad Prism 8 (v. 8.4.3, GraphPad Software Inc., California, USA) and significance levels are indicated as n.s. for non-significant differences, one asterisk (*) for $p<0.05$, two asterisks (**) for $p<0.01$, three asterisks (***).

Sensory data were first analyzed by means of 3-way ANOVA considering Samples (6), Judges (10), Replicates (2) and their relevant second order interactions as factors and sensory attributes as dependent variables. When the ANOVA showed a significant effect ($p<0.05$), the least significant difference (LSD) was applied as a multiple comparison test using the statistical software program STATGRAPHICS PLUS version 5.0 (Manugest KS Inc., Rockville, USA).

In order to examine the results from a multidimensional point of view, Principal Component Analysis (PCA) was then performed on sensory data averaged across judges and replicates (matrix Samples x Descriptors) using XLSTAT version 2019.2.2 (Addinsoft, Boston, MA, USA).

4.4.3 RESULTS

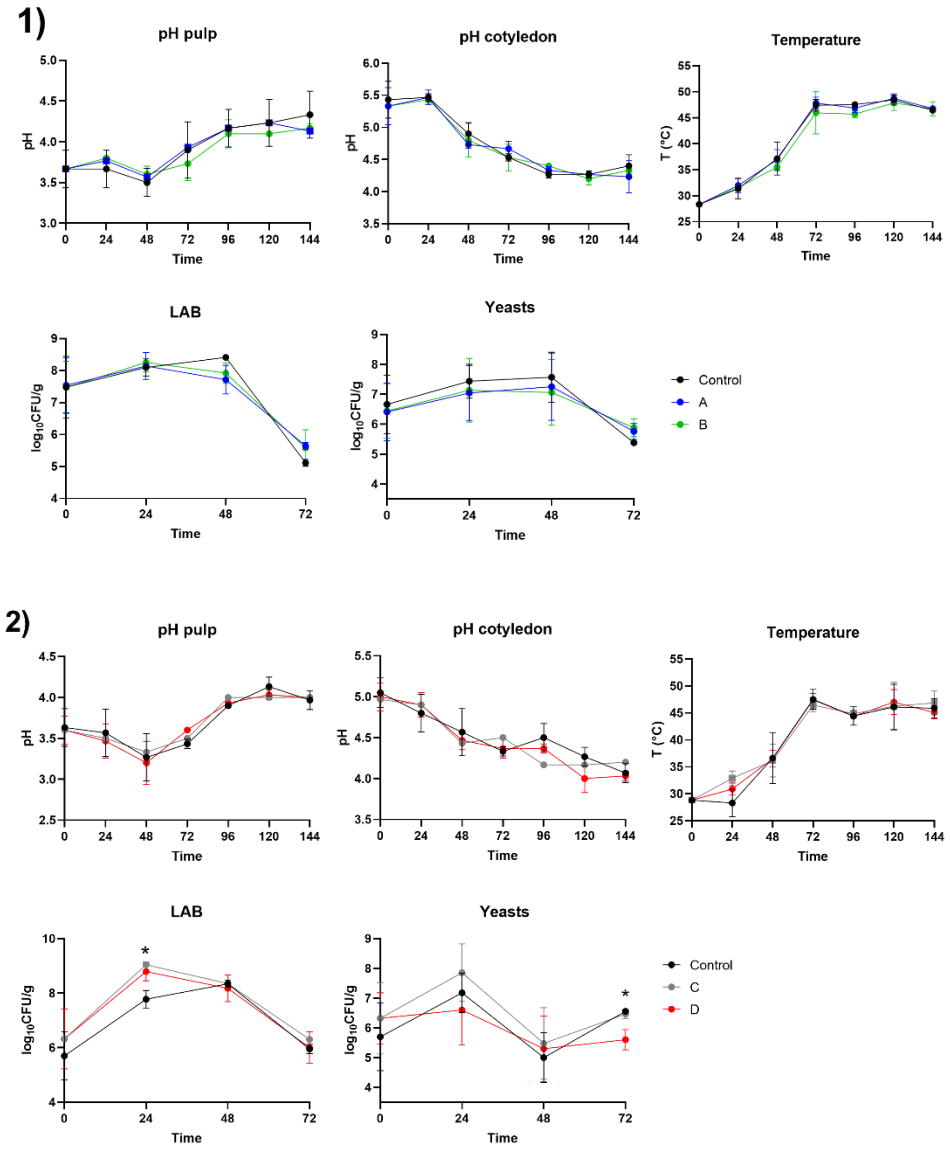
4.4.3.1 Fermentation parameters

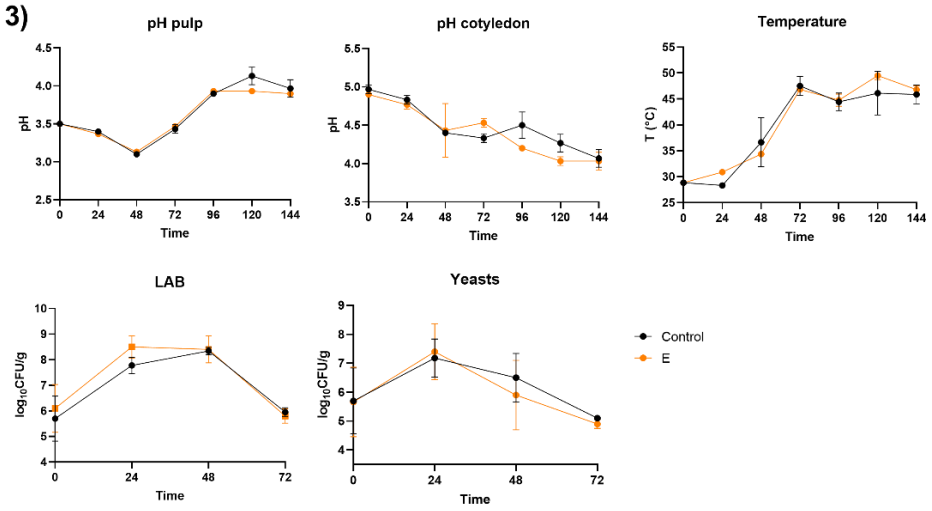
To study the progress of the fermentation, the temperature and pH of the cocoa samples were recorded daily. Counts of LAB and yeasts were also performed during the initial 72h of fermentation; after this period the LAB and yeasts were undetectable. The results for each fermentation setup are reported in Fig. 1. The inoculation did not have a significant impact on the temperature or pH of the pulp and cotyledon. Whereas the strain *L. fabifermentans* SAF13 did not significantly impact the total LAB or yeast counts, the inoculation of the strain *F. rossiae* SAF51 led to a significant increase in the total LAB count after 24h of fermentation, but this difference was not observed in the subsequent days. The inoculation of this strain after 48h of fermentation led to a significant decrease in total yeast count at the 72h mark. Similarly, the inoculation of the consortia of the two strains did not have a significant impact on the fermentation parameters considered.

The pH of the pulp did not significantly change during the first 24h of fermentation, despite the fermentation and organic acid production by the LAB. This aspect is related to the consumption of citric acid by the LAB, that has been known to raise the pH of the cocoa pulp at the beginning of the fermentation (Ouattara et al., 2016). The pH was the lowest at 48h, after which it increased to 4-4.5. The pH of the cotyledon on the other hand steadily decreased from the initial pH of 5-5.5 to about 4.5, matching the pH of the pulp. The temperature profile depended on the mixing of the fermenting mass, as it rose significantly after 48h when the first mixing was performed, to reach a temperature of 45-50°C that was maintained throughout the rest of the fermentation.

The LAB appeared to have their maximum growth at 24-48h from the beginning of the fermentation, whereas the growth of yeasts appeared to decrease earlier compared to LAB, that may be due to the higher sensitivity of yeasts to the stress factors such as the low pH and high temperature that start to take place after 48h of fermentation.

Fig. 1. Temperature, pulp and cotyledon pH and LAB and yeast counts for each inoculated species and different inoculation protocol: 1) *L. fabifermentans* at t0 (blue) and t0+t48 (green), 2) *F. rossiae* at t0 (grey) and t0+t48 (red), 3) co-culture at t0+t48 (orange) (Continues on next page)





4.4.3.2 Free amino acids profile

The results obtained are represented in Tab. 2 expressed as percentage of total protein. The chromatographs of the unfermented control and the control are shown in Fig. 2.

The total amount of free amino acids in the fermented samples was almost twice that of the unfermented sample. The inoculation of the strain *F. rossiae* SAF51 appears to promote the liberation of free amino acids when compared to the samples inoculated with the strain *L. fabifermentans* SAF13.

The fermentation protocol influenced the final amino acid concentration as well; when inoculated both at t0 and at t48, the total amount of amino acids was reduced.

Whereas the amount of some amino acids decreased with the fermentation (ASN, GLN, GABA, HIS, ARG), some remained unchanged (ASP, GLU, PRO), the amount of some amino acids increased significantly (THR, MET, LEU PHE, LYS). This amino acid liberation kinetic during cocoa fermentation has been reported previously by Kirchhoff et al. (1989a), that has also noted the elevated proportion between hydrophobic and acidic amino acids (1989b), that in our samples is approximately 70:15.

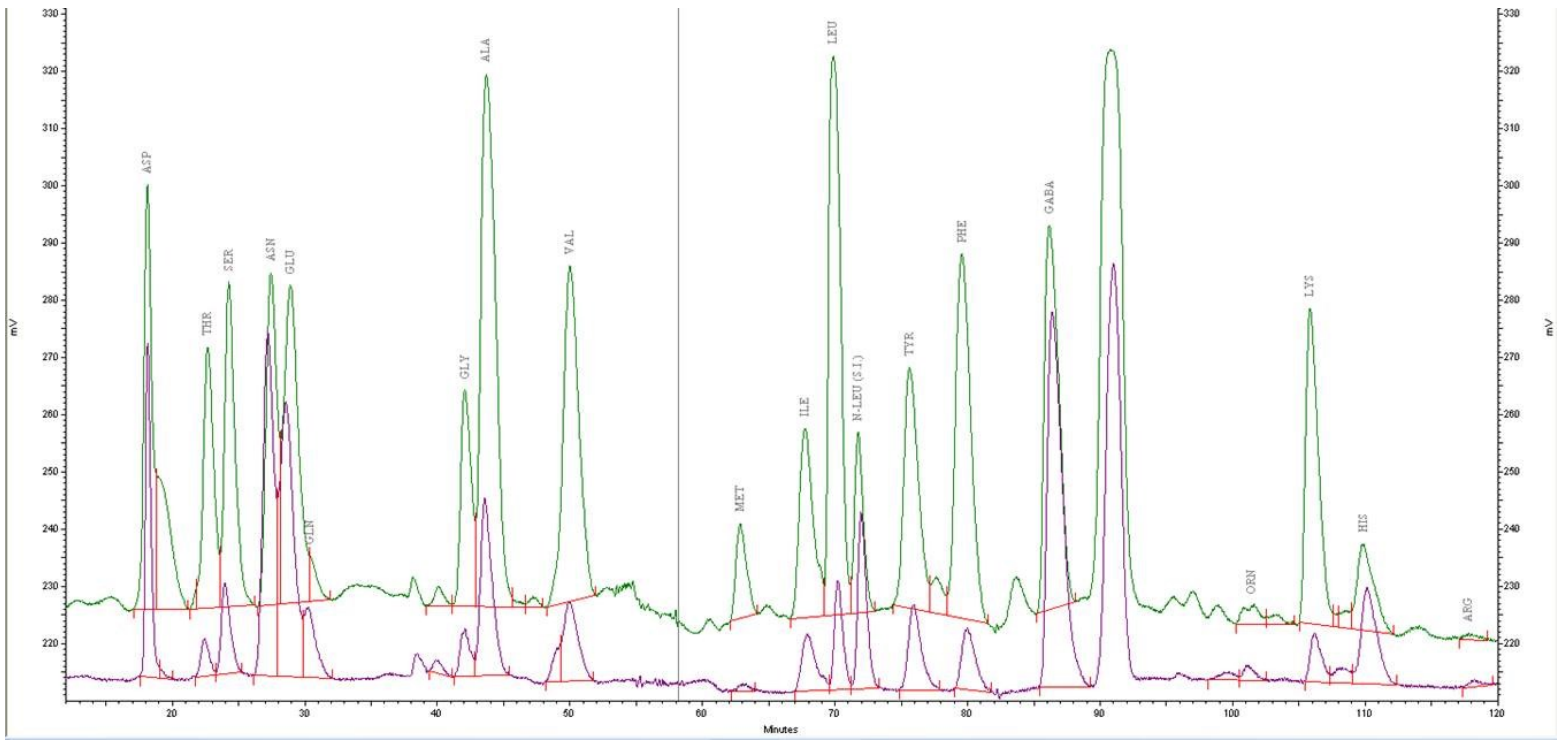
The amino acid profile of the samples inoculated with the strain *L. fabifermentans* SAF13 was similar to the one inoculated with the strain *F.*

rossiae SAF51, but when considering the relative proportion of the single amino acids, the samples inoculated with the strain SAF13 had a consistent abundance of ASN, GLU and ALA, whereas the sample inoculated with SAF51 contained a higher relative amount of ILE, TYR, ORN and LYS. The sample inoculated with the consortia *L. fabifermentans* SAF13 and *F. rossiae* SAF51 had a profile that closely resembled that obtained with *F. rossiae* SAF51 alone.

Tab.2 Free amino acids in dry cocoa beans expressed as percentage of the total protein content. A: inoculated with *L. fabifermentans* at t0; B: inoculated with *L. fabifermentans* at t0 and t48; C: inoculated with *F. rossiae* at t0; D: inoculated with *F. rossiae* at t0 and t48; E: inoculated with both strains at t0 and t48.

Amino acid	Unfermented control	Control	A	B	C	D	E
Asp	0.22	0.31	0.32	0.33	0.37	0.35	0.39
Thr	0.04	0.23	0.22	0.21	0.25	0.24	0.27
Ser	0.08	0.25	0.24	0.23	0.28	0.25	0.30
Asn	0.41	0.37	0.45	0.43	0.37	0.40	0.39
Glu	0.47	0.49	0.54	0.55	0.52	0.55	0.59
Gln	0.10	0.06	0.05	0.04	0.05	0.04	0.04
Gly	0.03	0.11	0.11	0.09	0.14	0.12	0.13
Ala	0.15	0.44	0.46	0.48	0.50	0.49	0.48
Cit	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Val	0.12	0.41	0.37	0.35	0.47	0.43	0.50
Met	0.01	0.08	0.05	0.03	0.12	0.07	0.11
Ile	0.10	0.24	0.22	0.21	0.29	0.27	0.32
Leu	0.13	0.79	0.75	0.78	0.85	0.82	0.87
Tyr	0.18	0.44	0.40	0.41	0.53	0.46	0.57
Phe	0.13	0.68	0.63	0.62	0.79	0.68	0.81
Gaba	0.48	0.45	0.37	0.40	0.46	0.43	0.47
Orn	0.02	0.01	0.02	0.01	0.03	0.03	0.03
Lys	0.08	0.44	0.39	0.36	0.53	0.47	0.52
His	0.26	0.22	0.18	0.18	0.23	0.20	0.24
Arg	0.03	0.03	0.02	0.01	0.01	0.02	0.02
Pro	0.13	0.22	0.22	0.18	0.24	0.20	0.28
Total	3.15	6.26	6.00	5.90	7.05	6.52	7.35

Fig.2 Chromatographs of the unfermented sample (purple) and fermented control (green).



4.4.3.3 Volatile molecules of cocoa mass

The amount in ppm of each volatile molecule detected is reported in Tab. 3. The flavour descriptors for each molecule are reported according to The Good Scents Company.

In general, the samples inoculated with the strain *L. fabifermentans* SAF13 present a higher total amount of volatiles when compared to the control, whereas the samples inoculated with the strain *F. rossiae* SAF51 contain a lower amount.

In the samples inoculated with *L. fabifermentans*, the compounds that increased the most, with concentrations more than twice that of the control, were 2,3-butanedion, hexanal, 2-heptanone and 2-nonanone. Furthermore, in the sample inoculated at t0 only, an increase of approximately 50% was recorded for 2-methyl butyric acid and 3-methyl butyric acid. On the other hand, a reduction was recorded for benzyl acetaldehyde, tetramethyl pyrazine and phenethyl acetate.

Considering the samples inoculated with the strain *F. rossiae* SAF51, a reduction in the majority of compounds was noted, that was especially pronounced in the sample inoculated both at t0 and at t48. 2-methyl butyric acid and 3-methyl butyric acid were the only compounds that registered an increase for both samples.

The sample inoculated with both strains has a similar profile to the one inoculated with the strain SAF51 only. An increase in the concentration of hexanal and 2-nonanone was reported, probably due to the high production of these compounds in presence of *L. fabifermentans* SAF13 as previously mentioned.

Significant differences were also observed for the acetic acid amount in the different samples. The fermentations inoculated with *F. rossiae* SAF51, as well as the one performed by the consortia of the two strains, had lower acetic acid amounts when compared to the control; the samples inoculated with the strain *L. fabifermentans* SAF13 on the other hand contained higher amounts of acetic acid.

Tab. 3. Amounts in ppm of volatile compounds measured from cocoa mass and respective flavour descriptors. A: inoculated with *L. fabifermentans* at t0; B: inoculated with *L. fabifermentans* at t0 and t48; C: inoculated with *F. rossiae* at t0; D: inoculated with *F. rossiae* at t0 and t48; E: inoculated with both strains at t0 and t48.

Volatile compound	Control	A	B	C	D	E	Descriptor
Methyl acetate	0.086	0.085	0.068	0.08	0.104	0.074	Green, fruity, fresh, rum and whiskey-like
2,3-Butandion	1.556	4.631	5.179	1.145	0.824	0.921	Sweet, buttery, creamy, milky
Acetic acid	231.979	276.219	314.842	130.573	67.649	88.608	Pungent, sour, overripe fruit
3-Methyl butanal	1.704	1.582	1.758	1.157	0.645	0.774	Fruity, green, chocolate, nutty, leafy, cocoa
2-Methyl butanal	0.565	0.402	0.629	0.463	0.196	0.211	Musty, rummy, nutty, cereal, caramel, fruity
Pentanal	0.378	0.437	0.541	0.107	0.11	0.257	Winey, bready, cocoa, chocolate notes
Hexanal	0.067	0.208	0.109	0.03	0.037	0.163	Green, woody, apple, grassy, orange
3-Methyl butyric acid	4.46	6.701	4.442	5.633	5.052	4.61	Cheesy, dairy, creamy, sweet, berry
2-Methyl butyric acid	2.403	3.709	2.566	3.605	3.829	3.571	Fruity, acidic, dairy, buttery
2-Heptanone	3.291	9.884	6.016	2.799	1.515	3.413	Cheese, fruity, coconut, waxy, green
2,3-Dimethyl pyrazine	0.066	0.073	0.066	0	0	0	Nutty, nut skin, cocoa, coffee, walnut
Butyrolactone	0.243	0.217	0.203	0.1	0.081	0.074	Milky, creamy with fruity afternotes
Benzaldehyde	0.57	0.733	0.8	0.545	0.419	0.425	Sweet, oily, almond, cherry, nutty, woody
Trimethyl pyrazine	0.402	0.391	0.357	0.345	0.29	0.31	Toasted, nutty, earthy, chocolate, coffee
Benzyl acetaldehyde	0.539	0.177	0.206	0.525	0	0.369	Green, melon, fruity, citrus
Tetramethyl pyrazine	5.22	3.788	2.962	5.926	5.26	3.638	Nutty, musty, cocoa, peanut, coffee notes
2-Nonanone	1.837	4.093	3.061	1.318	1.556	2.213	Cheesy, green, fruity, dairy, dirty, buttery
Nonanal	0.277	0.285	0.354	0.184	0.149	0.158	Citrus, cucumber, and melon rind
2-Phenylethanol	6.088	6.258	7.83	4.771	4.357	4.961	Floral, sweet, rosy and bready
Phenethyl acetate	4.857	3.161	4.248	2.298	1.898	1.843	Sweet, honey, floral, rosy

4.4.3.4 Sensory analysis

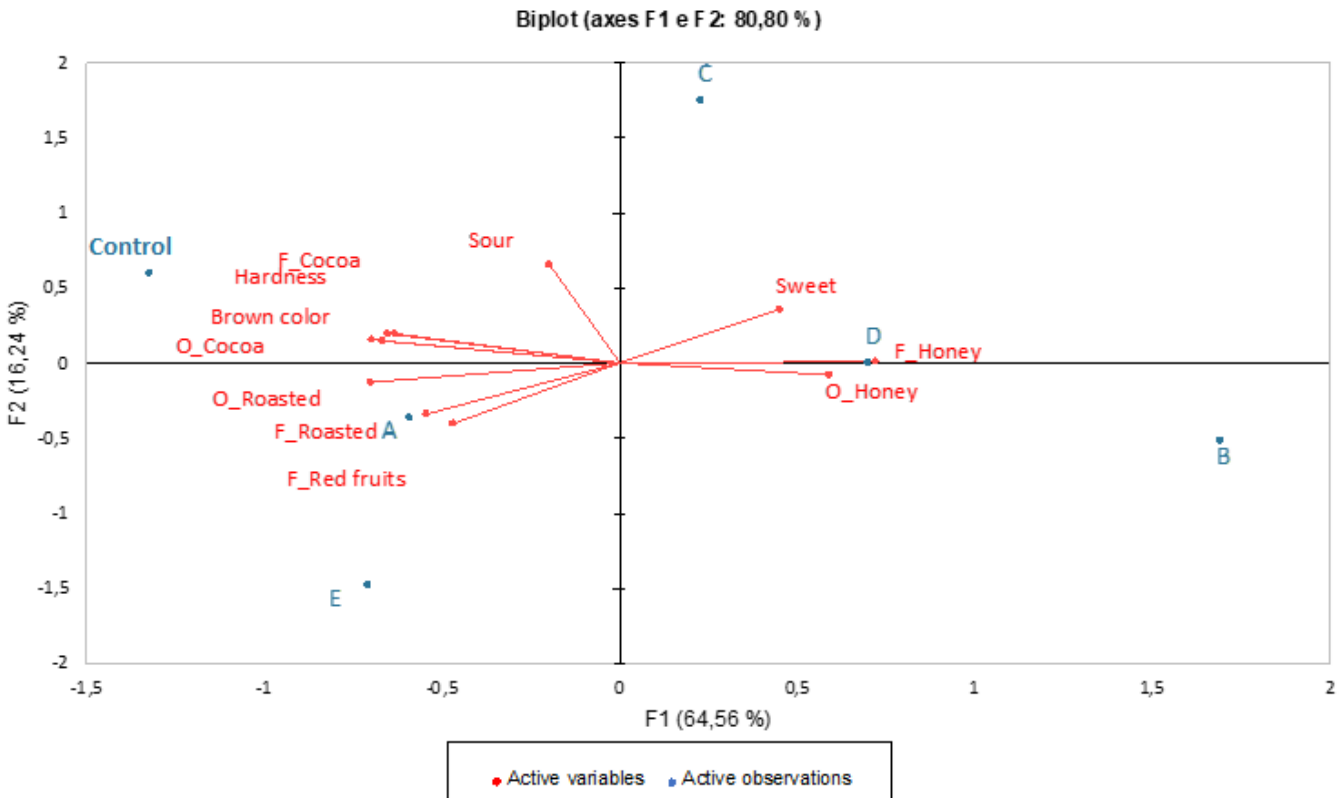
Three-way ANOVA results indicated that the panel of assessors was reliable (see Tab. S2, supplementary materials) and that all the sensory descriptors significantly ($p<0.05$) discriminated the samples (Tab. 4).

Since ANOVA results indicated that the mean scores for each sample given by the panel for each attribute could be assumed satisfactory estimates of the sensory profile of samples (Tab. S2), sensory data were averaged across assessors and replicates and submitted to PCA. According to correlation loadings plot, variables with less than 50% explained variance were left out from the analysis (i.e. red fruits odor, astringency and bitter). The biplot based on samples and the remaining variables are shown in Fig. 3. The variance explained by the first two principal components was 80,80%. Moving from left to right along the first component (explained variance 64,56%) of Fig. 3, the control sample and samples A and E were separated from the rest of the samples. The second component (explained variance 16,24%) distinguished samples C from E. The control sample (positioned in the upper left pane, Fig. 3) was mainly described by brown color, cocoa odor and flavor, sourness, and hardness and only partially by roasted odor and flavor. Samples A and E were mainly perceived with high intensity of roasted odor and flavor as well as red fruits flavor. These samples (A, E, control) had also the lowest intensity of sensory properties (honey odor and flavor and sweetness) located in the positive part of PC1 of Fig. 3 which mainly characterize samples D and B. Sample C (located in the upper right pane, Fig. 3) was mainly sour.

Tab. 4 Mean ratings (on a 9-point rating scale) obtained for the 6 dark chocolate samples for each sensory descriptor (*** significant at p<0.001; ** significant at p<0.01; * significant at p<0.05). Different letters by row indicate significant differences (p<0.05) according to LSD post hoc test. A: inoculated with *L. fabifermentans* at t0; B: inoculated with *L. fabifermentans* at t0 and t48; C: inoculated with *F. rossiae* at t0; D: inoculated with *F. rossiae* at t0 and t48; E: inoculated with both strains at t0 and t48.

Sensory dimension	**Sensory descriptor**	**Chocolate samples**					
		A	B	C	D	E	Control
Appearance	Brown color***	6,8d	5,2a	6,0c	5,8b	6,0c	7,0e
Odor	Cocoa ***	6,6b	5,9a	6,5b	5,9a	6,4b	6,6b
	Red fruits *	4,5bc	4,5bc	4,6bc	4,4ab	4,7c	4,2a
	Roasted ***	6,4c	5,4a	5,7b	5,6ab	6,2c	6,4c
	Honey **	4,8a	5,5b	4,7a	4,7a	4,5a	4,6a
Taste	Sweet ***	5,4c	5,4c	5,4c	5,5c	4,8a	5,1b
	Bitter ***	5,6c	5,4b	4,8a	4,6a	5,6c	5,6c
	Sour ***	5,6a	5,6a	6,2b	5,6ab	5,6a	6,0b
Flavor	Cocoa ***	6,5b	6,0a	6,3b	6,3b	6,3b	6,8c
	Red fruits ***	4,8c	4,0a	3,8a	3,8a	4,5bc	4,3b
	Roasted ***	6,0b	5,6a	5,6a	6,2c	7,0d	6,8d
	Honey ***	4,0a	5,6d	4,4b	4,8c	3,8a	3,8a
Mouthfeel	Hardness ***	6,4b	6,0a	6,4b	6,3b	6,5b	6,8c
	Astringency ***	4,8a	4,8a	4,8a	5,5b	5,3b	4,9a

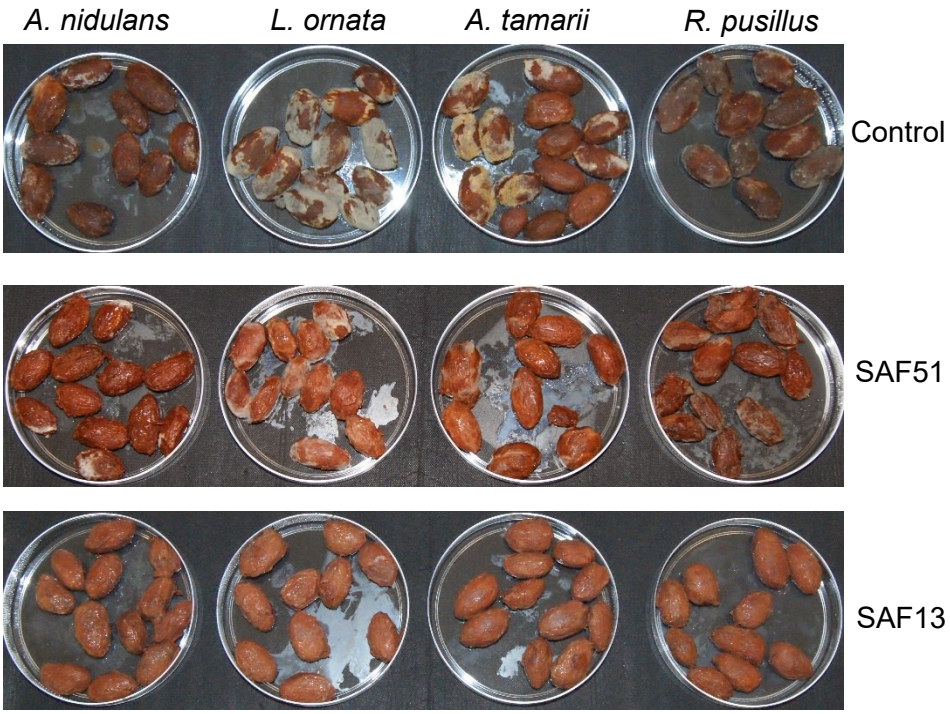
Fig. 3. Biplot obtained by the PCA model of chocolate sensory data. A: inoculated with *L. fabifermentans* at t0; B: inoculated with *L. fabifermentans* at t0 and t48; C: inoculated with *F. rossiae* at t0; D: inoculated with *F. rossiae* at t0 and t48; E: inoculated with both strains at t0 and t48. O indicates odour, F indicates flavour.



4.4.3.5 Mold growth inhibition

The results of the *in-situ* antifungal activity towards mold strains isolated from contaminated cocoa are represented in Fig. 4. Both strains showed some level of inhibition, but whereas the strain *F. rossiae* SAF51 delayed the growth of the four mold strains, with some growth observed for all four molds, the *L. fabifermentans* SAF13 completely inhibited the mold strains as no growth was observed after three days of incubation. On the other hand, all four mold species had a good ability to grow in the spontaneously fermented control.

Fig.4 Antifungal activity of strains *L. fabifermentans* SAF13 and *F. rossiae* SAF51 against four mold strains isolated from cocoa. Spontaneously fermented cocoa was used as control.



4.4.4 DISCUSSION

Controlled cocoa fermentations have received a strong interest from chocolate manufacturers and cocoa producers as a natural way to obtain specific flavour profiles without the need of using additives or flavourings. Although a lot of effort from the scientific community has been put into the study of different yeast species, the research is still lacking when it comes to LAB, especially in the study of minor species that do not dominate the fermentation but that may play a role in the flavour profile of the final product. Furthermore, there is a gap in knowledge about the optimal fermentation parameters to apply in a controlled fermentation and their role in the quality of the final product.

In this research we performed controlled fermentations of a fine flavour cocoa variety using two selected LAB strains, *F. rossiae* SAF51 and *L. fabifermentans* SAF13 as adjunct cultures. Two different inoculation setups were performed: the fermenting cocoa was inoculated either at t0 only, or both at the beginning and after the first mixing at t48; the second inoculation was performed because from the available scientific literature LAB are indicated to develop and perform at their optimum after the first stage dominated by yeasts.

The results obtained show that whereas the two strains do not significantly impact the fermentation parameters such as the temperature and the pH, they give different profiles when considering the final product. The fact that the fermentation parameters are similar to those of a typical well-fermented cocoa is a positive attribute, as they are crucial for the outcome of the fermentation process. Furthermore, there is no excessive acidity that has been seen as the main drawback of the presence of LAB in cocoa fermentations. Indeed, the sensory profile showed that the spontaneous control had one of the highest scores for acidity. This can be partially due to the heterofermentative phenotype of the LAB used, that produce different metabolites depending on the carbon source used, as opposed to homofermentative LAB that produce lactic acid exclusively. Furthermore, the GC-MS analysis on the cocoa liquor

showed a different concentration of acetic acid in the fermentation with each strain, so an interaction with the microbiota as a whole can also be hypothesised, given that acetic acid bacteria are the main producers of acetic acid.

Considering the free amino acids, it is unclear whether microorganisms have a direct activity on the cocoa proteins. Nevertheless, the addition of the adjunct cultures affected the free amino acid profile, regardless if it was due to the peptidases from the LAB that may be released after the cell death or from the creation of conditions closer to the optimum for the endogenous aspartic proteases and carboxypeptidases that have been suggested as the main enzymes that cleave and release amino acids in cocoa. Amino acids, di- and tripeptides are some of the most important precursors of flavour formation in chocolate. Not only do they participate in the Maillard reaction during roasting, giving the typical chocolate flavour, but they also go through degradation to produce aldehydes, ketones and other volatile molecules that have a significant impact in the flavour profile (Hinneh et al., 2018). Hydrophobic amino acids such as alanine, tyrosine and phenylalanine have been indicated as the main precursors of cocoa flavour formation (Rawel et al., 2019). Their concentration increases with cocoa fermentation, and the inoculation of the strain *F. rossiae* SAF51 appeared to promote their accumulation.

Because the cocoa liquor undergoes significant transformations to obtain chocolate, especially during conching, no direct correlation can be drawn between the volatile compounds detected in cocoa liquor and the flavour profile of the final product. Despite this, it is a useful method to highlight the potential activity of selected strains in a controlled fermentation. The addition of the adjunct cultures seemed to improve the flavour profile of the chocolate as they received a higher score for descriptors commonly used for fine chocolate such as honey and red fruits. The fermentation protocol also played an important role in all aspects considered, so it is important for future starter culture development to take this factor into consideration.

Furthermore, the cultures had a protective role to mold contamination, especially the strains *L. fabifermentans* SAF13 that inhibited completely the growth of mold. The possibility of using selected LAB to prevent mold contamination in cocoa has been previously investigated with good outcomes, so this aspect is an added value to the use of LAB in starter cultures for cocoa fermentation.

In conclusion, this work showed that LAB play a significant role to the flavour and quality of chocolate, and there are significant differences both between different strains and between different inoculation timepoints. Although their presence may not be essential to obtain a product with an acceptable taste, they can be an added value to the development of specific flavours that are desirable at an industrial level.

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4.4.5 References

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4.4.6 Supplementary materials

Tab. S1 List of sensory descriptors used to describe the samples with relevant definitions and reference standards.

Sensory dimension	Sensory Descriptor	Definition	Reference Standard
Appearance	Brown color	Characteristic perceived through the sense of sight referring to the brown color of chocolate	Minimum of the scale (score 1): milk chocolate (Lindt & Sprungli S.p.A.) Maximum of the scale (score 9): 100% criollo chocolate (Esselunga S.p.A)
Odor	Cocoa	Characteristic aroma of cocoa perceived orthonasally with the sense of smell	Cocoa powder Perugina (Nestlé Italiana S.p.A.)
	Red Fruits	Characteristic aroma of red fruits perceived orthonasally with the sense of smell	NeroNero extra dark chocolate with raspberries and almond granules (Novi S.p.A.)
	Roasted	Characteristic aroma of roasted/burnt perceived orthonasally with the sense of smell	100% criollo chocolate (Esselunga S.p.A)
	Honey	Characteristic aroma of honey perceived orthonasally with the sense of smell	Excellence dark with honey and almonds (Lindt & Sprungli S.p.A.)
Taste	Sweet	One of the basic tastes caused by sweet compounds (e.g. sugar) perceived in the oral cavity	Extra dark chocolate Perugina (Nestlé Italiana S.p.A.)
	Bitter	One of the basic tastes caused by bitter compounds (e.g. caffeine) perceived in the oral cavity	100% criollo chocolate (Esselunga S.p.A)
	Sour	One of the basic tastes caused by sour compounds (e.g. lemon) perceived in the oral cavity	100% criollo chocolate (Esselunga S.p.A)
Flavour	Cocoa	Characteristic flavour of cocoa perceived retronasally when swallowing	Cocoa powder Perugina (Nestlé Italiana S.p.A.)

	Red Fruits	Characteristic flavour of red fruit perceived retronasally when swallowing	NeroNero extra dark chocolate with raspberries and almond granules (Novi S.p.A.)
	Roasted	Characteristic aroma of roasted/burnt perceived retronasally when swallowing	100% criollo chocolate (Esselunga S.p.A)
	Honey	Characteristic flavour of honey perceived retronasally when swallowing	Excellence dark with honey and almonds (Lindt & Sprungli S.p.A.)
Mouthfeel sensations	Hardness	Degree of compression between the incisor teeth obtained prior to the breaking of the product	Minimum of the scale (score 1): milk chocolate (Lindt & Sprungli S.p.A.) Maximum of the scale (score 9): 100% criollo chocolate (Esselunga S.p.A)
	Astringency	Tactile sensation of dryness and puckering perceived in the oral cavity	100% criollo chocolate (Esselunga S.p.A)

Tab. S2. Effect of samples (6), judges (10), and replicates (2) on the 14 sensory descriptors considered (*** significant at p<0.001; ** significant at p<0.01; * significant at p<0.05; n.s. not significant).

Sensory dimension	**Sensory Descriptor**	Samples	Judges	Replicates	J X S	S X R	J X R
Appearance	Brown color	93,81***	1,83n.s.	0,10n.s.	1,83*	0,57n.s.	1,83n.s.
Odor	Cocoa	13,45***	2,26*	0,00n.s.	1,52n.s.	0,38n.s.	0,75n.s.
	Red Fruits	3,15*	6,84***	3,31n.s.	1,97*	1,39n.s.	0,97n.s.
	Roasted	27,59***	7,05***	1,79n.s.	2,27**	0,62n.s.	1,46n.s.
	Honey	4,97**	1,77n.s.	0,73n.s.	1,18n.s.	0,36n.s.	0,45n.s.
Taste	Sweet	10,08***	2,31*	0,00n.s.	1,45n.s.	2,91*	1,56n.s.
	Bitter	30,31***	8,09***	0,06n.s.	1,14n.s.	1,15n.s.	1,53n.s.
	Sour	15,47***	8,65***	2,06n.s.	1,62n.s.	2,23n.s.	0,47n.s.
Flavour	Cocoa	10,04***	3,71**	0,24n.s.	1,12n.s.	0,24n.s.	1.95n.s.
	Red Fruits	11,25***	5,03***	6,41*	1,40n.s.	1,01n.s.	1,47n.s.
	Roasted	44,90***	2,42*	0,13n.s.	1,03n.s.	0,39n.s.	1,18n.s.
	Honey	33,47***	4,46***	0,06n.s.	1,13n.s.	1,98n.s.	2,01n.s.
Mouthfeel sensations	Hardness	9,85***	4,76***	0,53n.s.	1,49n.s.	0,50n.s.	0,80n.s.
	Astringency	17,30***	11,19***	0,69n.s.	1,16n.s.	0,46n.s.	2,04n.s.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this PhD work we presented a polyphasic approach for the design of starter or adjunct cultures for two different fermented food products.

Strains isolated from food fermentations showed a good ability to resist to stress conditions associated with the fermentation process. Selected LAB strains had the ability to delay or inhibit the growth of mold, both *in-vitro* and *in-situ*. The application of the starter cultures had clear positive effects on the final product when compared to the spontaneous fermentation.

The first part of the study was focused on the fermentation of a spelt-based sourdough bread. Spelt is an ancient grain which, after decades of marginal cultivation, has been upgraded for its reputation as a healthy and sustainable food. This research was carried out to overcome the poor performance of spelt in bread making. The study of the LAB composition of spontaneously fermented spelt highlighted the dominance of *Pediococcus pentosaceus*, especially in the late stages of backslopping. Spelt sourdough revealed to be a good source of robust and highly performant strains for a potential sourdough starter.

Strains belonging to *Weissella cibaria* and *W. confusa* were studied to be used in conjunction with *P. pentosaceus*. The first important step in the utilization of these two species was the safety evaluation, that is a prerequisite for the industrial use of any bacterial strain. Since the two species are not included in the QPS list, a combined genotypical and phenotypical approach was used to evaluate their potential pathogenicity, but no activities of concern were discovered. On the other hand, the two species have interesting probiotic and pro-technologic activities that make them useful in starter culture, for this reason, we suggest the two species to be included in the QPS list.

The non-conventional yeast *Kazachstania unispora* also possessed interesting characteristics for a potential use in the bakery sector. The species had a higher resistance to stress conditions when compared to a commercial strain of *Saccharomyces cerevisiae*, and had a good ability to ferment glucose

and fructose, whereas the maltose-negative phenotype makes it a good candidate to be used in conjunction with LAB in co-culture fermentations as they do not compete for the carbon source. The application of the selected strains to a spelt-based bread resulted in an improved texture and shelf-life.

In the second part of the project, we assessed the role of the addition of LAB in cocoa fermentation. Selected strains isolated from spontaneously fermented cocoa had a significant impact on the flavour profile of the final product, as well as on its safety as a strong inhibition towards cocoa related mold strains was demonstrated.

In conclusion, this research showed that selected strains from fermented foods have a great potential not only in obtaining a product in a reliable and reproducible way, but also in maintaining or even improving the beneficial properties and desirable flavour profile of traditional fermented products. However, further efforts must be made for their real application at industrial level, also considering the related economic aspects. This implies a careful evaluation of the costs for the production of biomass, compared to the final quality of the product that can be obtained and marketed. In both food sectors investigated, there is great interest in the use of new starter cultures, to satisfy increasingly detailed consumer needs. For this reason, future prospects are linked to following up research in direct contact with manufacturing companies.

6. Scientific production

Publications with impact factor

Quattrini, M., **Korcari, D**., Ricci, G., & Fortina, M. G. (2019). A polyphasic approach to characterize *Weissella cibaria* and *Weissella confusa* strains. *Journal of Applied Microbiology*, *128*(2), 500–512. https://doi.org/10.1111/jam.14483

Korcari, D., Ricci, G., Quattrini, M., & Fortina, M. G. (2019). Microbial consortia involved in fermented spelt sourdoughs: Dynamics and characterization of yeasts and lactic acid bacteria. *Letters in Applied Microbiology*, *70*(1), 48–54. https://doi.org/10.1111/lam.13241

Korcari, D., Ricci, G., Capusoni, C., & Fortina, M. G. (2021). Physiological performance of *Kazachstania unispora* in Sourdough Environments. *World Journal of Microbiology and Biotechnology*, *37*(5). https://doi.org/10.1007/s11274-021-03027-0

Korcari, D., Secchiero, R., Laureati, M., Marti, A., Cardone, G., Rabitti, N. S., Ricci, G., & Fortina, M. G. (2021). Technological Properties, shelf life and consumer preference of spelt-based sourdough bread using novel, selected starter cultures. *LWT*, *151*, 112097. https://doi.org/10.1016/j.lwt.2021.112097

Korcari, D., Ricci, G., Fanton, A., Emide, D., Barbiroli, A., & Fortina, M. G. (2021). Exploration of *Lactiplantibacillus fabifermentans* and *Furfurilactobacillus rossiae* as potential cocoa fermentation starters. Submitted to Journal of Applied Microbiology

Korcari, D., Fanton, A., Ricci, G., Rabitti, N. S., Laureati, M., Hogenboom, J., & Fortina, M. G. (2021) Fine cocoa fermentation with selected Lactic Acid

Bacteria: fermentation performance and impact on chocolate flavour. To be submitted to Food Research International.

National and International Conferences

Korcari, D., Quattrini, M., Ricci, G., Fortina, M. G. (2019). Microbial communities associated with spelt sourdoughs. Microbial Diversity Conference 2019, Catania, Italy, 25-27th Semptember 2019. Poster presentation.

Korcari, D. (2019). Investigating the biodiversity in spontaneous fermentations as a source of high-performance microorganisms. XXIV Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Firenze, Italy, 11-13th September 2019. Poster presentation.

Korcari, D. (2021). Investigating the biodiversity in spontaneous fermentations as a source of high-performance microorganisms. I virtual Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Palermo, Italy, 14-15th September 2021. Oral presentation.