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Exploring the Microbial Community of Traditional Sourdoughs to Select Yeasts and Lactic Acid Bacteria †

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Abstract: Sourdoughs represent an awesome example of ecosystem in which yeasts and lactic acid bacteria (LAB) interact with each other, defining the characteristics of the final product in terms of composition, texture, taste and flavor. Therefore, the identification of dominant yeasts and LAB involved in the fermentation process can lead to the selection of starters with suitable fermentation aptitude and capable of producing desired aromas and/or aromatic precursors. In this work, two sourdoughs samples (A and B) for Panettone production were collected from an artisan bakery. Yeasts and bacteria were isolated at different fermentation steps on selective agar media. A total of 120 isolates were obtained and firstly characterized by conventional microbiological methods. Afterward, genomic DNA was extracted from the cultures, and $(GTG)_5$-PCR fingerprinting analysis was carried out to reduce the redundance among the isolates. Representative yeasts and LAB strains, having a unique profile, were identified by sequencing the D1/D2 domain of the 26S rRNA and the 16S rRNA genes, respectively. The results highlighted the occurrence of *Kazachstania humilis* and *Fructilactobacillus sanfranciscensis* in both sourdoughs. Among LAB, also some other strains belonging to *Lactobacillus* genus were found. Moreover, *Saccharomyces cerevisiae* and *Staphylococcus* spp. strains were detected in sample B. In this study, a pool of yeasts and LAB strains for producing starter cultures with specific technological traits for sourdoughs production was obtained.

Keywords: sourdoughs; starter culture; sequencing

1. Introduction

Sourdough is a microbial ecosystem of lactic acid bacteria (LAB) and yeasts in a matrix of mainly cereal flour and water [1]. Although yeasts and LAB mainly occur, other microorganisms may be present, such as acetic acid bacteria and those coming from the raw material and/or from the process [2]. The microbial community of sourdough is continuously kept active due to the back-slopping procedure that characterize the manufacturing process. Since millennia, humankind has exploited microbial diversity associated to different food matrices and used their microbial community as natural starter culture. The research of new starter cultures is of great interest in the scientific community because they can be exploited as driver of innovation in the food and beverage field [3]. In this preliminary work, we investigated the microbial community of two sourdoughs samples used for producing traditional Panettone. Yeasts and LAB were isolated and identified with the final aim

to select strains capable of producing desired aromas and/or aromatic precursors, in order to exploit them as new starter cultures.

2. Methods

2.1. Collection of Samples

Two sourdoughs, used to produce artisanal Panettone, were collected from a traditional bakery of the Emilia Romagna region (Italy). Above them, other samples were collected during two different back-slopping methods started from the sourdoughs, respectively (the former was refreshed three times, the latter only two). In particular, from the two sourdoughs MA and MB, the samples IMPA, IMPB and FINA, FINB were obtained. They were taken, respectively, from the final dough before the leavening and at the end of leavening before cooking. Except for the sourdough back-slopping procedure, the same ingredients were used, and all the samples were prepared and collected on the same day.

2.2. Yeasts and LAB Isolation

To obtain microbial isolates, 10 g of each sample was collected and mixed with 90 mL of physiological solution (9 g/L of NaCl) in a Stomacher bag and homogenized for 2 min. From the homogenate samples serial dilutions from 10^{-2} to 10^{-5} were made, and appropriated dilutions were plated in duplicate on selective agar media. Yeasts were isolated onto WL nutrient medium (OXOID) and Yeast peptone dextrose agar (YPDA; 10 g/L yeasts extract, 10 g/L peptone, 20 g/L dextrose and 20 g/L agar) containing 0.125 g/L of chloramphenicol. LAB was isolated onto de Man Rogosa Sharpe (MRS, OXOID) and Sourdough Bacteria Agar (SDB; 6 g/L trypticase, 3 g/L yeast extract, 20 g/L maltose, 3 mL of 10% TWEEN 80, 15 g/L fresh yeast extract, 12 g/L agar). These media were supplemented with 0.125 g/L cycloheximide. In total, 120 isolates were chosen and purified by streaking on the correspondent isolation medium. Basic phenotypic tests, such as the observation of the morphology of the colonies for yeasts and Gram staining and catalases for bacteria, respectively, were done. After the molecular characterizations yeasts and bacteria strains have been preserved in the Unimore Microbial Culture Collection (UMCC) in accordance with standard procedures [4].

2.3. DNA Extraction and Preliminary Characterization

Genomic DNA from yeast and bacterial cultures was extracted followed the protocols of Hoffman and Winston [5] and Ausubel et al. [6], respectively. Restriction Fragment Length Polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region and 5.8S gene of rDNA was performed on yeasts by using the oligonucleotide primers ITS1 and ITS4 and *Hae*III (Thermofisher) restriction endonucleases [7]. To type all the isolates, $(GTG)_5$-PCR fingerprinting was conducted according to Lattanzi et al. [8].

2.4. Species Assignment

To obtain a species assignment, the D1/D2 domain of 26S rDNA of the yeasts showing different $(GTG)_5$ clusters was amplified with the primers NL1 and NL4, following the protocol of Kurtzman et al. [9]. PCR products were cleaned before sequencing by MWG Biotech Company (Ebersberg, Germany). Regarding the bacteria, amplification of 16S rRNA gene of isolates with different $(GTG)_5$ profiles was performed using primers 27f and 1490r [8], and the PCR products were sequencing as described before. All the sequences obtained were processed with Chromas and Bioedit software and then aligned in GeneBank with the Blast program [10] to determine the closest known relatives.

3. Results and Discussion

The microbial density found in MA and MB samples showed values around 7–7.5 ufc/mL. In the other samples, the population was slightly higher with values of about 8 ufc/mL. After a preliminary

microbiological screening, 60 yeast isolates and 25 bacterial isolates (Gram +) were selected and furtherly investigated (Tables 1 and 2).

3.1. Yeasts Molecular Characterization

$(GTG)_5$-PCR fingerprinting technique allowed to group isolates in 15 different clusters. The results of the typing and D1/D2 domain sequencing are reported in Table 1. Sourdough A shows a strong dominance of *Kazachstania humilis* species, with only one sample identified as a group-species *K. humilis*/*K. parahumilis*. This homogeneous result was already observed in traditional sourdoughs [2,11–13]. In sourdough B, four strains belonged to the *S. cerevisiae* species, while the other strains belonged to *K. humilis* species (Table 1). These two species represent typical yeast interactions found in other traditional sourdoughs [12,14]. Furthermore, as demonstrated by Fujimoto et al. [15], the reduced fermentation of sourdough could favor the dominance of *S. cerevisiae*, as happened in sourdough B.

Table 1. RFLP analysis, $(GTG)_5$-PCR and sequencing of D1/D2 domain of the 26S rDNA gene of the yeast strains isolated from sourdough's samples.

Sample	Strain	RFLP-PCR ITS-5.8S *Hae*III Restriction Enzyme	$(GTG)_5$-PCR Cluster	D1/D2 Domain of 26S rRNA Gene Sequence Comparison Species, Accession N. (% identity)
MA	LMA2, **LMA3** *	400-230	A	*K. humilis* NG_055707(99%)
	LMA1, LMA4, **LMA5**,	400-230	-	*K. humilis* NG_055707(98%)/ *K. pseudohumilis* NG_058281(98%)
	LMA6, LMA7, LMA8	400-230	-	
	LMA9, LMA10	400-230	B	*K. humilis* NG_055707(99%)
IMPA	**LIA1**, LIA9, LIA10	400-230	C	*K. humilis* NG_055707(99%)
	LIA2, LIA3, LIA4,	400-230	D	
	LIA5, LIA7	400-230	D	*K. humilis* NG_055707(99%)
	LIA6, LIA8	400-230	E	*K. humilis* NG_055707(99%)
FINA	**LFA1**	400-230	F	*K. humilis* NG_055707(100%)
	LFA2, **LFA3**, LFA4,	400-230	G	*K. humilis* NG_055707(97%)
	LFA5	400-230	G	
	LFA6	400-230	H	*K. humilis* NG_055707(99%)
	LFA7, LFA8	400-230	A	
	LFA9, LFA10	400-230	C	
MB	**LMB1**	400-230	-	*K. humilis* NG_055707(100%)
	LMB2, LMB3, LMB6,	320-230-180-150	I	
	LMB7, LMB10	300-230-180-150	I	
	LMB4, LMB5	400-230	L	*K. humilis* NG_055707(100%)
	LMB8	400-230	M	
	LMB9	320-230-180–150	N	*S. cerevisiae* NG_042623 (100%)
IMPB	LIB1, LIB4, **LIB**7,	320-230-180-150	I	*S. cerevisiae* NG_042623(100%)
	LIB10	320-230-180-150	I	
	LIB2, LIB3, **LIB5**,	400-230	O	*K. humilis* NG_055707(100%)
	LIB6, LIB8, LIB9	400-230	O	

FINB	**LFB1**, LFB2, LFB3,	320-230-180-150	P	*S. cerevisiae* NG_042623(100%)
	LFB4, LFB6	320-230-180 -150	P	
	LFB5, LFB7, LFB8,	400-230	O	
	LFB9	320-230-180-150	O	
	LFB 10	320-230-18-150	Q	*S. cerevisiae* NG_042623(100%)

*: the sequenced strains are reported in bold.

3.2. Bacteria Molecular Characterization

The bacteria isolates belonged to the LAB group with the exception of four strains, identified as *Staphylococcus* spp. species (Table 2). The 25 strains subjected to $(GTG)_5$-PCR fingerprinting highlighted 14 different clusters. The results showed the presence of heterofermentative obliged bacteria except for *Lactobacillus plantarum*, a facultative heterofermentative bacterium, normally colonizing sourdough continuously back-slopped and maintained at a low temperature [1] (Table 2). *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*) was found in both sourdoughs with a major presence in the A samples. This species is typical of a traditional sourdough environment and is selected precisely by its process condition [16], allowing its presence both in dominant and non-dominant conditions [15,17–19]. The presence of *Furfurilactobacillus rossiae* (formerly *Lactobacillus rossiae)* in both sourdough samples is interesting, this species was recently described by Corsetti et al. [20] and subsequently studied for its fermentative properties by Di Cagno et al. [21]. *F. rossiae* can be found both individually and in association, as a dominant species, with other LAB species, as shown in sample IMPA (Table 2). The species *Leuconostoc citreum* and *Leuconostoc mesenteroides*, found in both sourdoughs, are typical of the last stages of Panettone process [19]. Moreover, their presence, along with *L. plantarum*, *F. rossiae* and *Fr. sanfranciscensis* can be explained with the low temperature of the process [1]. The presence of *Lactobacillus plantarum* in sourdough is also widely described both as a sub-dominant and dominant species [19,22]. Less common but explainable [22] is the presence of *Lentilactobacillus parabuchneri (*formerly *Lactobacillus parabuchneri*), commonly found in beer or dairy products. In contrast to sample A, sourdough B shows a contamination of *Staphylococci,* probably of human origin (Table 2) and explainable by the manipulation and the back-slopping of the dough.

Table 2. $(GTG)_5$-PCR cluster and sequencing of the 16S rRNA gene of the bacterial strains isolated from sourdough's samples.

Sample	**Strain**	**$(GTG)_5$-PCR** Cluster	**16S rRNA Gene sequence comparison** Species, accession N. (% identity)
MA	**BMA2, BMA8, BMA10 ***	A	*L. sanfranciscensis* X76327 (99%)
IMPA	**BIA2**, BIA3	B	*L. parabuchneri* LC383822 (100%)
	BIA5, BIA8, **BIA10**	C	*L. rossiae* NR_112758 (99%)
	BIA6	D	*L. citreum* NR_041727 (99%)
	BIA7	E	*L. mesenteroides* NR_074957 (100%)
	BIA9	F	
FINA	**BFA1**	G	*L. plantarum* CP039121 (100%)
	BFA2	H	*L. citreum* NR_041727 (99%)
MB	**BMB5**	I	*L. sanfranciscensis* X76327 (99%)
	BMB7	L	*L. sanfranciscensis* X76327 (99%)
	BMB10	-	*S. epidermidis* LN681574 (99%)
IMPB	**BIB6**	M	*S. epidermidis* LN681574 (100%)
	BIB7, BIB8	N	*L. rossiae* NR_112758 (99%)
	BIB9	-	
	BFB1	M	*S. epidermidis* LN681574 (99%)

	BFB3**, BFB8**	O	*L. citreum* NR_041727 (99%)
FINB	**BFB6**	P	*L. mesenteroides* NR_074957 (100%)
	BFB7	-	*S. hominis* subsp. *novobiosepticus* MF678884 (100%)

*: the sequenced strains are reported in bold.

4. Conclusions

The constant search for new starter cultures with optimal and increasingly precise performance is leading to a rediscovery of the great microbial diversity present in food processes such as sourdoughs. In addition, the new food trends are increasingly asking industries for natural, eco-friendly products with beneficial properties. The molecular characterization of these two sourdoughs (composed of samples MA, IMPA, FINA and MB, IMPB, FINB) microbiota showed the presence of the main microorganisms normally colonizing this environment and can allow the selection of yeasts and LAB to be used as new starters.

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