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Diversity and technological potential of lactic acid bacteria of wheat flours

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ABSTRACT

Lactic acid bacteria (LAB) were analysed from wheat flours used in traditional bread making throughout Sicily (southern Italy). Plate counts, carried out in three different media commonly used to detect food and sourdough LAB, revealed a maximal LAB concentration of approximately 4.75 Log CFU g^{-1}. Colonies representing various morphological appearances were isolated and differentiated based on phenotypic characteristics and genetic analysis by randomly amplified polymorphic DNA (RAPD)-PCR. Fifty unique strains were identified. Analysis by 16S rRNA gene sequencing grouped the strains into 11 LAB species, which belonged to six genera: *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella*. *Weissella cibaria*, *Lactobacillus plantarum*, *Leuconostoc pseudomesenteroides* and *Leuconostoc citreum* were the most prevalent species. The strains were not geographically related. Denaturing gradient gel electrophoresis (DGGE) analysis of total DNA of flour was used to provide a more complete understanding of the LAB population; it confirmed the presence of species identified with the culture-dependent approach, but did not reveal the presence of any additional LAB species. Finally, the technological characteristics (acidifying capacity, antimicrobial production, proteolytic activity, organic acid, and volatile organic compound generation) of the 50 LAB strains were investigated. Eleven strains were selected for future *in situ* applications.

1. Introduction

Cereal based foods have been key components of human diets for thousands of years. They remain a main source of nutrition, particularly in developing and overpopulated countries (Blandino et al., 2003). Indeed, the history of several cultures is directly defined by cereals and, consequently, many populations are identified by the cereals they eat with Mediterranean people referred to as "wheat people" (Gifford and Baer-Sinnot, 2007). Historically, millennia B.C. wheat was among the most important crops grown in the Mediterranean basin. Following mass migrations, its cultivation underwent a huge expansion, resulting in its production worldwide (Toderi, 1989).

Wheat is critical in the Mediterranean diet: it provides approximately one-third of the daily protein and energy requirements (~2400 kcal) for an adult (Cannella and Piredda, 2006). For this reason, wheat surpasses other cereals in terms of the number of hectares dedicated to its cultivation worldwide (Gifford and Baer-Sinnot, 2007).

Wheat kernels are naturally contaminated with microorganisms (Corsetti et al., 2007), which may then be present in flours. The microorganisms that contaminate cereals are generally concentrated in the outer layers of kernels, and they tend to stay in fractions rich in bran during milling. Consequently, flour obtained from milling should theoretically contain a lower bacterial load than caryopses, but the subsequent conditioning phase can increase flour's microbial content (Berghofer et al., 2003). Among the microbial populations associated with wheat kernels, protechnological lactic acid bacteria (LAB) are often detected (Corsetti et al., 2007) and are also found in wheat flours (Russo et al., 2010). Because raw materials are not commonly subjected to thermal treatments before the fermentation stage, they provide a source of living, active LAB during the production of baked goods. This is especially important for baked goods prepared with sourdough: in this mixture of flour and water, LAB enable the production of lactic and acetic acid (Salovaara, 1998). LAB that contribute to wheat flour fermentation can also come from the equipment used in the milling and/or production process (Berghofer et al., 2003).

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Although a number of studies have identified and characterized LAB of mature sourdoughs, only a few have analysed the microbial ecology of raw materials used in the production of baked goods.

Understanding the microbial composition during fermentation at the species and strain level is particularly important for the correct management of sourdough. Microorganisms within flours may be essential in ensuring sourdough contains the correct proportion of bacteria and yeasts as well as the optimal ratio between heterofermentative and homofermentative species of LAB. The overall sourdough microbial composition is especially important in the early stages of development, when typical sourdough LAB are not present at high levels and the acidity does not inhibit the undesired microbiota.

Investigation of LAB can be difficult, especially in raw materials subjected to uncontrolled conditions. Although wheat flour contains all of the nutrients required for the growth of several microorganisms, including the nutritionally fastidious LAB, its low water content does not permit LAB growth. Indeed, within flours, several LAB species are found in a dormant state (Corsetti et al., 2007), from which their subsequent culturing in specific media is not guaranteed, even if optimal incubation conditions are applied. Therefore, to reveal the complete microbial population within flours, total DNA needs to be investigated using culture-independent methods.

To characterise the LAB populations within wheat flours that are commonly used to make traditional Sicilian breads, the specific objectives of this study were to: (i) enumerate, isolate and genetically differentiate (at the strain level) LAB found in flour samples collected from throughout Sicily; (ii) perform bacterial image analysis on all flour samples using a culture-independent tool; (iii) evaluate the technological capabilities of LAB by examining their acidifying abilities in a flour extract system, proteolytic activity, production of antibacterial and antifungal compounds, and generation of chemical compounds.

2. Materials and methods

2.1. Sample collection

Thirteen wheat (*Triticum durum* and *Triticum aestivum*) flour samples (Table 1) were collected from several bakeries throughout Sicily (southern Italy) from January to May 2012, immediately before the mature sourdough was refreshed. Samples were chosen for their typical characteristics and/or origin. Many of the flours are unique to local Sicilian milling, while others are also commercially available outside Sicily. Flour samples were transferred into sterile Stomacher bags and placed into a portable cooler. Once in the laboratory, all samples were refrigerated until analysed.

2.2. Microbiological analysis

Each sample (15 g) was suspended in Ringer's solution (135 mL) (Sigma–Aldrich, Milan, Italy), homogenised in a stomacher (BagMixer® 400; Interscience, Saint Nom, France) for 2 min at maximum speed, and then serially diluted. Decimal dilutions were plated on three agar media: MRS (Oxoid, Milan, Italy) (generic for rod LAB) pour plated and incubated anaerobically at 30 °C for 48 h; M17 (Oxoid) (generic for coccus LAB) pour plated and incubated anaerobically at 30 °C for 48 h; Sour Dough Bacteria (SDB) (Kline and Sugihara, 1971), specific for sourdough LAB, spread plated and incubated aerobically at 30 °C for 48 h. To avoid fungal growth, cycloheximide (10 mg mL^{-1}) was added to all media. Microbiological counts were performed in triplicate.

Statistical analyses were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial data were analysed using a generalised linear model (GLM) that included the effects of sample; the Student "*t*" test was used for mean comparison. The *post-hoc* Tukey method was applied for pairwise comparison. Significance level was $P < 0.05$.

2.3. LAB isolation and phenotypic grouping

Four colonies of Gram-positive [Gregersen KOH method (Gregersen, 1978)] and catalase negative bacteria of differing morphologies were isolated following their growth on the three media from the LAB count experiments (from the highest plated dilution), to represent the diversity of LAB, and subsequently transferred into the corresponding broth media. The isolates were then purified with successive sub-culturing. Isolates used in subsequent analyses were stored at −80 °C in broth containing 20% (v/v) glycerol.

Phenotypic characterisation was carried out to obtain an initial grouping of isolates. The cell morphology type of LAB isolates was determined using an optical microscope. Rod- and coccus-shaped LAB cultures were further grouped based on cell disposition. Specifically, rod LAB were sub-grouped according to their growth at 15 and 45 °C and ability to produce CO_2 from glucose. The final grouping test was carried out with the same growth media used for isolation, but with citrate removed because its fermentation by certain LAB can result in gas formation. Furthermore, M17 contained glucose in place of lactose, and SDB was prepared with glucose instead of maltose. For the assay, LAB were inoculated into test tubes sealed with H_2O agar (2%, w/v). After incubation for 48 h, LAB were scored positive for CO_2 production if a rising of the agar cap was detected, indicating the LAB had an obligate heterofermentative metabolism. LAB strains that scored negative in the assay were

Table 1
Samples of wheat flours used to produce traditional sourdough breads in Sicily.

Sample	Species	Flour	Commercial name	Company	Geographical origin
F2	*T. durum*	Durum wheat semolina	Local grain	Salvia	Partinico (PA)
F3	*T. durum*	Durum wheat semolina	Linea Blu – Sicilian grain	Landro S.n.c. company – Manata Santo & C.	Misilmeri (PA)
F4	*T. aestivum*	Soft wheat flour	Nazionale Archilli	Grandi Molini Italiani	Pachino (SR)
F5	*T. aestivum*	Soft wheat flour	Mister Sprint	Molitoria Sanpaolo S.p.A.	Siracusa
F6	*T. durum*	Whole meal durum wheat semolina	Linea Rossa	Landro S.n.c. company – Manata Santo & C.	Misilmeri (PA)
F7	*T. aestivum*	Soft wheat flour	Terra	Selezione Casillo S.r.L.	Corato (BA)
F9	*T. durum*	Durum wheat semolina	Local grain	Molini Gattuso	Castronovo di Sicilia (PA)
F10	*T. durum*	Durum wheat semolina	Divella	F. Divella S.p.A.	Rutigliano (BA)
F11	*T. durum*	Durum wheat semolina	Local grain	Granaio del Sole	Palermo
F23	*T. durum*	Durum wheat semolina	Local grain	Molino Ivana	Roccapalumba (PA)
F26	*T. durum*	Durum wheat semolina	Local grain	Molino Ancona	San Giovanni Gemini (AG)
F30	*T. durum*	Durum wheat semolina	Local grain	Molini Roccasalva Giorgio & C.	Modica (RG)
F33	*T. durum*	Durum wheat semolina	Local grain	Molini Grillo & C. S.a.S.	Marsala (TP)