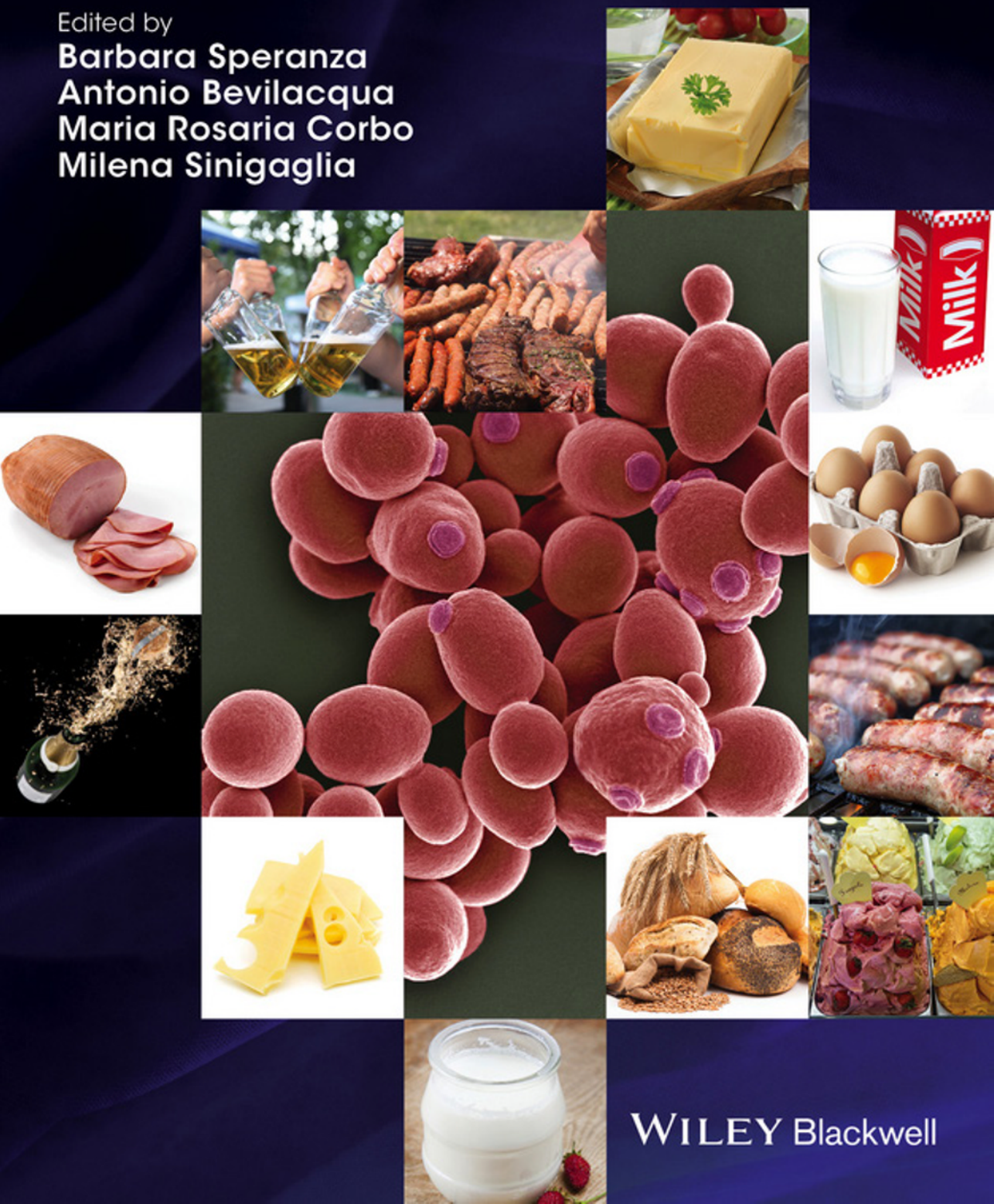
STARTER CULTURES IN FOOD PRODUCTION

Edited by
Barbara Speranza
Antonio Bevilacqua
Maria Rosaria Corbo
Milena Sinigaglia



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Barbara Speranza
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Maria Rosaria Corbo
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Milena Sinigaglia
University of Foggia, Italy

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Editorial Offices
9600 Garsington Road, Oxford, OX4 2DQ, UK
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Contents

List of contributors

Clelia Altieri
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Ersilia Arace
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Francisco Noé Arroyo-López
Food Biotechnology Department, Instituto de la Grasa (CSIC), Spain

Salvatore Augello
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Antonietta Baiano
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Antonio Bevilacqua
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Pietro Buzzini
Department of Agricultural, Food and Environmental Science, Industrial Yeasts Collection DBVPG, University of Perugia, Italy

Giuseppe Calò
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Daniela Campaniello
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Angela Capece
Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Italy

Vittorio Capozzi
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Emanuela Ciuffreda
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Maria Rosaria Corbo
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Roniele P. Cordeiro
Department of Food Science, University of Manitoba, Canada

Philippe Dantigny
Université de Brest, LUBEM, ESIAB, France

Fabio de Stefano
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Barbara Di Maggio
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Simone Di Mauro
Department of Agricultural, Food and Environmental Science,
Industrial Yeasts Collection DBVPG, University of Perugia, Italy

Francesca Fuccio
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Mariangela Gallo
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Pilar García
Instituto de Productos Lácteos de Asturias–Consejo Superior de Investigaciones
Científicas (IPLA-CSIC), Spain

Antonio Garrido-Fernández
Food Biotechnology Department, Instituto de la Grasa (CSIC), Spain

Maria Clara Iorio
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Rufino Jiménez-Díaz
Food Biotechnology Department, Instituto de la Grasa (CSIC), Spain

Takashi Kuda
Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Japan

Martina Loi
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Fernando B. Luciano
School of Agricultural Sciences and Veterinary Medicine,
Pontifícia Universidade Católica do Paraná, Brazil

Renata E.F. Macedo
School of Agricultural Sciences and Veterinary Medicine,
Pontifícia Universidade Católica do Paraná, Brazil

Beatriz Martínez
Instituto de Productos Lácteos de Asturias–Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Spain

Noemi Monacis
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Labia Irène I. Ouoba
Consultant – Senior Research Scientist, London, UK

Marianne Perricone
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Leonardo Petruzzi
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Stefano Pignatiello
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Italy

Angela Racioppo
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Ana Rodríguez
Instituto de Productos Lácteos de Asturias–Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Spain

Patrizia Romano
Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Italy

Patricia Ruas-Madiedo
Instituto de Productos Lácteos de Asturias–Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Spain

Pasquale Russo
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Borja Sánchez
Instituto de Productos Lacteos de Asturias–Consejo Superior de Investigaciones Cientificas (IPLA-CSIC), Spain

Luca Settanni
Department of Agricultural and Forest Sciences, University of Palermo, Italy

Milena Sinigaglia
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Giuseppe Spano
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Barbara Speranza
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Sanna Taskila
Chemical Process Engineering, University of Oulu, Finland

Benedetta Turchetti
Department of Agricultural, Food and Environmental Science, Industrial Yeasts Collection DBVPG, University of Perugia, Italy

Chibuike C. Udenigwe
Department of Environmental Sciences, Dalhousie University, Canada

Preface

As classically defined, starter cultures are living microorganisms or defined combinations that are deliberately used for the fermentation of raw material and applied to elicit specific changes in the chemical composition and sensorial properties of the substrate.

Due to their vital role in the manufacturing, flavour and texture development of fermented foods, the awareness that starter cultures are of great industrial significance is a well-established fact. Once mainly used in the dairy industry, nowadays the addition of selected starter cultures has spread to all fermented food products (meat, sourdough, vegetables, wine, fish), where their use ensures a correct and predictable process and avoids fermentation arrests or the production of undesired metabolites. Depending on the type of action and the product to be obtained, a starter should fit some predetermined selection criteria. In the last 20 years, the selection of starter cultures for food has been an emerging topic; the main issue has been the evaluation of the technological traits of autochthonous strains, with the main aim of selecting some biotypes adapted to the different raw materials. Many papers can be easily found in the literature dealing with these topics; namely, with the quali-quantitative composition of the lactic microflora from dairy products, vegetables, meat, sourdough and so on. These reports clearly underline the industrial importance of starter cultures (mainly lactic acid bacteria) for the manufacture of fermented food products, and different selection protocols are described.

Over the last decade new concepts have emerged, including the use of functional starter cultures, the use of genomic approaches to select promising starter cultures, the use of new kinds of starter (like fungi) and the use of microorganisms as non-conventional starters to manage the waste from the food industry. These emerging ideas could be the future as well as a tentative practical application of starter cultures in the food industry, as they could offer a solution to the increasing demand for new ways to give functional/added value to some traditional food products.

Therefore, the main goal of this book is to describe the most recent insights around this topic, through 19 chapters covering all new concepts related to this issue. For example, advances in genetics and molecular biology have recently provided opportunities for genomic studies of starter cultures, aimed to design and improve industrially useful strains. The selection of new starter cultures is beginning to take advantage of pangenomic, based on a comparison of the complete genome sequences of a number of members of the same species; pangenomic

does in fact open up an array of new opportunities for understanding and improving industrial starter cultures and probiotics. These include understanding the formation of texture and flavour in food products; understanding the functionality of probiotics; and providing information that can be used for strain screening, strain improvement, safety assessment and process improvement.

Another growing issue is starter attenuation through physical methods. Attenuated starters are lactic acid bacteria that do not have the ability to produce acid during fermentation, but contain enzymes that can influence food quality (for example, during cheese ripening). Besides heat treatment, different methods to achieve attenuation have been studied, including freezing and thawing, freeze or spray drying, lysozyme treatment, high-pressure treatment, use of solvents, and natural and induced genetic modification. To the best of our knowledge, little information is actually available about both pangenomic and starter attenuation, so an overview of what has been done and what can be done could help the scientific and academic community.

Moreover, even if starter microorganisms have mainly useful and positive aspects, could they negatively affect human health and well-being? Some starter cultures can produce both biogenic amines and other toxic compounds; this aspect is often overlooked and we have devoted a chapter to this lesser-known issue.

Lactic acid bacteria are the main microorganisms responsible for fermentation and are consequently used as starter cultures by definition; surprisingly, fungal starters have also been reported as a promising means in some fermentations and appear to survive, and even grow, in stressful environments. However, neither their role nor the mechanism facilitating their survival and growth under these conditions is completely understood. A special focus on this new concept of starter cultures could be appreciated, especially if applied to the management of wastes from the food industry.

In this book we have tried to update and collate information and research carried out on various aspects of these innovative features. We have also devoted an entire second section to analysing and describing what has been done and what is known about different fermented food products: sourdough and cereal-based foods, table olives and vegetables, dairy and meat products, fish, wine and ethnic foods. One special focus is the selection of functional *Bacillus* starter cultures for alkaline fermentation.

We are grateful to all the contributing authors who accepted our invitation to write this book. We are happy to bring numerous foreign authors on board, and offer our thanks to Francisco Noé Arroyo-Lopez, Philippe Dantigny, Takashi Kuda, Renata E.F. Macedo, Labia Irène I. Ouoba, Ana Rodriguez, Patricia Ruas-Madiedo and Sanna Taskila and their colleagues, who have given an international dimension to this project. We are also grateful to our Italian colleagues Clelia Altieri, Pietro Buzzini, Angela Capece, Vittorio Capozzi, Leonardo Petruzzi and Luca Settanni, and to everyone who collaborated with them.

We also want to thank the editorial staff of John Wiley & Sons for their guidance in all the aspects that made the publication of this book possible.

We hope the book will be utilized by researchers, students, teachers, food entrepreneurs, agriculturalists, ethnologists, sociologists and people in general who are interested in fermented foods and starter cultures.

The editors
June 2016

CHAPTER 1
Lactic acid bacteria as starter cultures

Clelia Altieri, Emanuela Ciuffreda, Barbara Di Maggio and Milena Sinigaglia
Department of the Science of Agriculture, Food and Environment, University of Foggia, Italy

Introduction

Starter cultures have a basic role: to drive the fermentation process. Concomitantly, they contribute to all the characteristics of products, as well as to their sensorial and safety characteristics. Therefore, the introduction of starter cultures has undoubtedly improved the quality of products and the standardization of the industrial process.

A very important aspect is to have a good knowledge of the metabolic properties required to improve a specific product and to select useful microbial strains. Nevertheless, the limited number of already selected and studied strains that are also able to possess highly technological properties, as well as the constant risk of bacteriophage attacks, are stimulating research into new starter strains, in order to obtain higher quality and product diversification, in response to more and more aware consumers.

General aspects of starter cultures

The production of fermented foods today is based on the use of starter cultures, for example lactic acid bacteria (LAB), which initiate fast acidification of raw material. The great advantage of starter cultures is that they can provide controlled and predictable fermentation.

Starter cultures of LAB can contribute to microbial safety or offer one or more technological, organoleptic, nutritional or health advantages. Examples are LAB that produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins or useful enzymes, or that have probiotic properties (Leroy and De Vuyst 2004).

While starter cultures, chosen on the basis of their good safety and 'functional' characteristics, can benefit the consumer, they must first be able to be

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manufactured under industrial conditions (Saarela *et al.* 2000). Safety aspects of LAB include specifications such as origin, non-pathogenicity, certain metabolic activities (e.g. deconjugation of bile salts), toxin production, haemolytic potential, side effects in human studies (i.e. systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer) and epidemiological surveillance of adverse incidents in consumers (post-market). Functional aspects can be related to viability and persistence in the gastrointestinal (GI) tract, survival at low and high pH and in the presence of bile salts, hydrophobic properties, antibiotic resistance patterns, immunomodulation, and antagonistic and antimutagenic properties. Technological aspects concern growth at different sodium chloride (NaCl) amounts, temperatures, pH values, acidifying ability and metabolism (arginin deamination, esculin hydrolysis, acetoin production) and the ability to produce adequate flavour/texture.

With regard to the effect of salting, the addition of NaCl is a common practice in most fermented dairy foods, and also affects the growth of starter bacteria. Most LAB are partially or fully inhibited by levels of NaCl higher than 5%. However, it is evident that salt tolerance is a strain-dependent characteristic, thus this criterion is important in starter selection (Powell *et al.* 2011).

LAB starters are primarily used because of their ability to produce lactic acid from lactose and for consequent pH reduction, leading also to important effects like inhibition of undesirable organisms, improvement of sensorial and textural properties, as well as contribution to health benefits. A major role of starter cultures in dairy production is the degradation of peptides generated by the coagulant to small peptides and amino acids. Starter cultures are also capable of degrading caseins and converting amino acids to a range of flavour compounds. However, since many of the proteolytic enzymes are intracellular, flavour development in maturing cheese also depends on the release of the enzymes from starter cultures into the cheese matrix through cell lysis. Cell lysis, and the consequent release into the cheese matrix of intracellular enzymes, particularly peptidases and amino acid-degrading enzymes, is an important characteristic for both general protein degradation and also the control of bitterness. Autolysis results from the enzymatic degradation of the bacterial cell wall by indigenous peptidoglycan hydrolases released into the growth medium, although it is still unclear how this process is controlled in the cell. The process is highly strain dependent and is also influenced by factors such as the nutrient status of the growth medium and environmental conditions (Lortal and Chapot-Chartier 2005).

Generally, in maturing cheese there is a positive relationship between the period of starter culture autolysis and the flavour-forming reactions, involving not only proteolysis but also lipolysis. Consequently, various screening assays using buffers or model cheese and milk solutions have been proposed to select highly autolytic strains for use in cheese manufacture. Lysis positively influences the ripening and flavour of the cheese, but the type of peptidases is also very

important, in particular since low peptidase activities and low lytic properties produce bitter cheese. One of the most successful strategies to counteract this defect involves the use of LAB with high peptidase activities, particularly Pep N.

For these reasons, the use of good starter cultures can ensure the safety, quality and acceptability of both traditional and innovative fermented dairy products.

Types of starter cultures

In practice starter cultures may be categorized as mesophilic or thermophilic, according to the incubation and manufacturing temperatures under which they are used. Mesophilic cultures grow and produce lactic acid at optimal levels, at a moderate temperature (about 30 °C), whereas thermophilic cultures optimally function at a higher temperature (about 42 °C). Examples of mesophilic dairy starter cultures are the species *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc lactis*. On the other hand, the most thermophilic LAB species are *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactobacillus helveticus*.

Nevertheless, the most common classification of starter cultures is based on the complexity of the culture and the way it is reproduced. All starter cultures available today are derived in one way or another from **natural starters** (or artisanal starters) of undefined composition (i.e. containing an undefined mixture of different strains and/or species). For some types of products, natural starters have been replaced by commercial **mixed-strain starters** (MSS), derived from the 'best' natural starters and reproduced under controlled conditions by specialized institutions and commercial starter companies, then distributed to the industries that use them to build up bulk starter or for direct vat inoculation. Natural starter cultures and commercial MSS, because of their long history, are called **traditional starters** (Limsowtin *et al.* 1996) as opposed to **defined strain starters** (DSS). DSS are usually composed of only a small number of selected strains and allow greater control over the composition and properties of the cultures. Table 1.1 shows a summary of culture types.

Traditional cultures contain many strains of many microbial species, sometimes including yeasts and moulds as well as bacteria; they all contribute biochemically to the complexity (and the variability) of the final product (Powell *et al.* 2011). Therefore, traditional starter preparation methods are still in use for some particular or traditional products, and have been adapted to a limited industrial scale. Industrial-scale production requires starters that give reproducible performance and are free of undesirable organisms. These goals are difficult to achieve using traditional methods. Thus, DSS have replaced traditional starters in industrial-scale production because of their optimized, highly reproducible performance and their high phage resistance.

Table 1.1 Culture types and their preparation.

Types of starter cultures		Description
Traditional starters	Natural starters	Low cost. Undefined composition. Highly variable composition and performance. Prone to undesirable contamination; microbiologically hazardous
Traditional starters	Mixed-strain starters (MSS)	Undefined composition. Variable composition and performance. With careful handling and some quality control testing, these are still in limited use, but have largely been replaced by laboratory-maintained cultures
Defined strain starters (DSS)		Defined composition, usually composed of only a small number of strains. This gives a high degree of control over starter performance parameters and product properties, as long as strains are carefully selected and managed

Traditional starters: Natural starters

The production of natural starters is derived from the ancient practice of backslopping (the use of an old batch of a fermented product to inoculate a new one) and/or by application of selective pressures (heat treatment, incubation temperature, low pH) (Carminati *et al.* 2010). No special precautions are used to prevent contamination from the environment, and the control media and culture conditions during starter reproduction are very limited. As a result, natural starters are continuously evolving as undefined mixtures composed of several strains and/or species (Carminati *et al.* 2010).

Natural starters are an extremely valuable source of strains with desirable technological properties (antimicrobials, aroma production); for example, they are considered to be highly tolerant to phage infection because they are reproduced in the presence of phages, which leads to the dominance of resistant or tolerant strains (Carminati *et al.* 2010). Also they seem to be advantaged by microbial interactions; in fact, many strains show limited acid-production ability when cultivated as pure cultures (Parente and Cogan 2004).

Traditional starters: Mixed-strain starters (MSS)

MSS, obtained by careful selection of natural starters, are maintained, propagated and distributed by starter companies and research institutions (Parente and Cogan 2004). Like artisanal starters, MSS contain an undefined mixture of strains that differ in their physiological and technological properties (Parente and Cogan 2004).

When undefined cultures are propagated under controlled conditions with a minimum of subcultures, the stability of their composition and performance is greatly improved in comparison to natural strains (Stadhouders and Leenders 1984).

The composition of MSS is undefined, but their reproduction under controlled conditions reduces the intrinsic variability associated with the use of natural starters (Limsowtin *et al.* 1996).

The traditional method for reproduction of MSS, which requires several transfers to build up the bulk starter by using small amounts of stock cultures, has been replaced by the use of concentrated cultures for the inoculation of the bulk starter tank, thus minimizing the need for transfers within the factory and the risk of fluctuations in starter composition and activity (Carminati *et al*. 2010).

Defined strain starters (DSS)

DSS are composed of one or more strains (the dominant species of the traditional product) and are selected, maintained, produced and distributed by specialized companies. Since the strains and/or species ratio in DSS is defined, their technological performance is extremely reproducible and this is a desirable property. In fact, in recent years DSS have replaced traditional starters (Carminati *et al*. 2010). However, as a consequence of the limited number of strains used, a phage infection may cause disruption of lactic acid fermentation. Furthermore, with the subsequent loss of natural microbial diversity, maintenance of the typical features is difficult. Nevertheless, examination of the key properties of each strain (i.e. genetic or biochemical features, growth and acid-production characteristics) can lead to the rational mixing of strains, in order to formulate a culture with a desirable set of properties (Carminati *et al*. 2010).

DSS usually have no defects of flavour, and have a distinctive trait of 'cleaner' aroma and flavour. In order to increase control over their nature and attain a flavour as close as possible to the traditional one, industrial companies are making increasing use of flavour-enhancing adjunct cultures; DSS cultures are added at low levels to the starter, and may themselves be defined or undefined (Powell *et al.* 2011).

Metabolism of lactic acid bacteria

LAB are important in many food fermentations because they contribute to sensory characteristics and preservative effects (Holzapfel 1995) with their physiological features such as substrate utilization and metabolic capabilities. Some LAB are homofermentative and produce lactic acid as the main product of glucose fermentation, while others are heterofermentative and produce carbon dioxide and ethanol in addition to lactic acid (Blandino *et al*. 2003).

It is clear that LAB adapt to various conditions and change their metabolism accordingly. This may lead to significantly different end product patterns, thus LAB metabolism is essential to study when selecting new starter strains.

Lactose metabolism

Lactose, a disaccharide composed of glucose and galactose, is the only free-form sugar present in milk (45–50 g/L). The main pathways for lactose metabolism are shown in Figure 1.1.

The transport of lactose into a cell requires energy. In the lactococci, this energy is sourced via energy-rich phosphoenolpyruvate (PEP), an intermediate of the glycolytic pathway. This is part of a transport mechanism referred to as the phosphoenolpyruvate phosphotransferase system (PEP-PTS), in which the lactose is phosphorylated as it is transported across the cell membrane. Once inside the cell, phosphorylated lactose is hydrolysed by the enzyme phospho-β-galactosidase to glucose and galactose-6-phosphate. The glucose moiety enters the glycolytic pathway, and galactose-6-P is converted into tagatose-6-phosphate via the tagatose pathway. Both sugars are cleaved by specific aldolases into triose phosphates, which are converted to pyruvic acid at the expense of nicotinamide adenine dinucleotide (NAD^+). For continued energy production, NAD^+ must be regenerated. This is usually accomplished by reducing pyruvic acid to lactic acid (Poolman 1993).

In other dairy starter bacteria, including *Strep. thermophilus*, leuconostocs, lactobacilli and bifidobacteria, lactose transport appears to be via a specific protein (a permease) that translocates the lactose into the cell without modification,

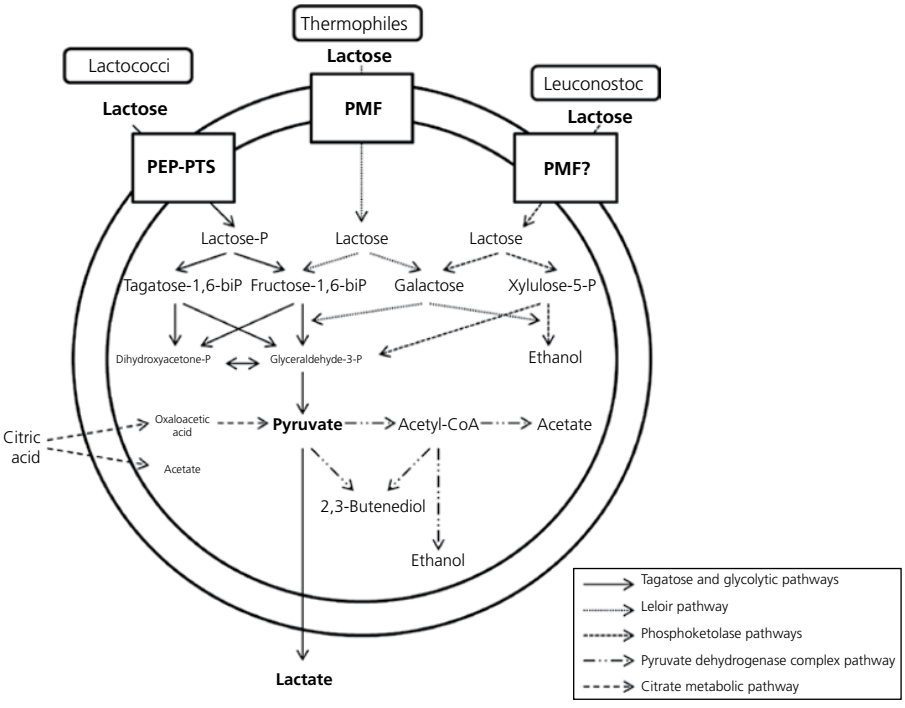

Figure 1.1 General pathways for carbohydrate catabolism by lactic acid bacteria.

although in many of these organisms the exact nature of the system used is still unclear. The lactose is then hydrolysed by β-galactosidase to glucose and galactose (Powell *et al.* 2011). The glucose moiety enters the glycolytic pathway, but galactose is excreted from the cells and accumulates in milk or cheese. Thermophilic lactobacilli that do not excrete galactose and *Lb. helveticus* strains utilize the Leloir pathway to metabolize galactose, while *Lb. delbrueckii* subsp. *bulgaricus* and most strains of *Strep. thermophilus* cannot metabolize galactose. This is a problem in cheese manufacture, since residual sugar can be metabolized heterofermentatively by other bacteria.

It is not known how lactose is transported in cells by *Leuconostoc* species or heterofermentative lactobacilli; however, lactose is known to be hydrolysed by β-galactosidase (Huang *et al.* 1995).

The galactose moiety is transformed into glucose-6-phosphate (Leloir pathway) and, together with glucose, is metabolized through the phosphoketolase pathway.

Lactic acid and ethanol, respectively, are formed during this metabolism to regenerate NAD^+; however, where lactococci are fermenting galactose or lactose at growth-limiting rates, products other than lactic acid can be formed from pyruvate. The enzyme pyruvate formate lyase is able to convert pyruvate to formate, acetate, acetaldehyde and ethanol under anaerobic conditions and at high pH (>7.0). Under aerobic conditions and at pH 5.5–6.5, pyruvate can be converted to acetate, acetaldehyde, ethanol and the minor products acetoin, diacetyl and 2,3-butanediol via the multienzyme pyruvate dehydrogenase complex.

Citrate metabolism

Citrate metabolism in LAB has been reviewed by Hugenholtz (1993). Milk contains 0.15–0.2% citric acid, but not all LAB can metabolize it. However, *Leuconostoc* species, Cit^+ *Lb. lactis* subsp. *lactis* and facultative heterofermentative lactobacilli do metabolize citric acid (Palles *et al.* 1998).

Many LAB use citrate as a substrate for cometabolism with sugars like glucose, fructose, lactose or xylose, providing NADH (citrate + 2 [H]/lactate + acetate + CO_2) (Hache *et al.* 1999) not directly as an electron acceptor, but as a precursor of acetate and oxaloacetate, which will be the final electron acceptor after being decarboxilated. Citrate metabolism is important in *Lc. lactis* and *Ln. mesenteroides* strains, which are often used in the dairy industry.

The latter organism was called *Streptococcus diacetylactis* in the old literature and more recently *Lc. lactis* subsp. *lactis* biovar *diacetylactis*. This name has no taxonomic status and the correct way to refer to it is citrate-utilizing (Cit^+) *Lc. lactis* subsp. *lactis*. Cit^+ strains of *Lc. lactis* differ from non-citrate-utilizing (Cit^-) strains because they contain a plasmid that encodes the transport of citrate. *Leuconostoc* species and Cit^+ *Lc. lactis* subsp. *lactis* strains utilize citric acid and lactose simultaneously and under certain conditions can derive energy via metabolism of citric acid.

Citric acid is transported into the cell by a citric acid permease, which is plasmid encoded in lactococci and *Leuconostoc* (Vaughan *et al.* 1995), and metabolized to pyruvic acid without generation of NADH. The result is an excess of pyruvic acid, which can be used to produce lactic acid to regenerate NAD^+, or in other reactions that regenerate NAD^+ and/or $NADP^+$.

The enzymes involved in these reactions are inducible and their expression is influenced by sugar concentrations and pH; in fact, a low amount of sugar and low pH favour diacetyl/acetoin formation.

Historically, there was a debate on which pathway was the most important. Evidence now clearly prefers the route via α-acetolactate, since α-acetolactate can be detected as an intermediate in cultures producing diacetyl and an α-acetolactate synthase has been identified in several LAB (Hugenholtz 1993).

Diacetyl contributes to typical yoghurt flavours and is produced by chemical decomposition of α-acetolactate (non-enzymatic). This reaction is favoured by aeration and low pH. Acetoin and/or 2,3-butanediol is produced in much larger amounts than diacetyl, but does not contribute to the aroma (Marshall 1987). Hugenholtz (1993) describes the use of genetic engineering to construct strains of lactococci able to produce high levels of diacetyl.

Nitrogen metabolism

Nitrogen metabolism by starters has an enormous impact on their activity and on cheese quality. LAB are fastidious microorganisms and are unable to synthesize many amino acids, vitamins and nucleic acid bases. Depending on the species and the strain, LAB require from 6 to 14 different amino acids (Kunji *et al.* 1996).

The proteolytic system of LAB is very complex and consists of three major components: a cell-wall bound proteinase that promotes extracellular casein degradation into oligopeptides, then peptide transporters that move peptides into the cytoplasm, where finally there are various intracellular peptidases that degrade peptides into smaller molecules and amino acids (Liu *et al.* 2010).

Proteolysis is a major event in cheese ripening: the proteolytic system of primary starter and secondary microflora contributes to the production of hundreds of flavour compounds through the synthesis of low-molecular-weight peptides and amino acids and their subsequent catabolism.

Free amino acids and peptides in cheese can contribute to flavour either directly or indirectly and with positive or negative effects. Cheese flavour development has been the subject of a comprehensive review (Smit *et al.* 2005). A major negative effect of proteolytic products is bitterness, which is believed to be caused by hydrophobic peptides ranging in length from 3 to 27 amino residues (Lemieux and Simard 1992). These peptides are believed to be generated

from casein principally by the joint action of chymosin and LAB proteinases (Broadbent *et al.* 1998) and can be hydrolysed to non-bitter peptides and amino acids by LAB peptidases. In particular, the enzymatic degradation of proteins (caseins) leads to the formation of key flavour components, which contribute to the sensory perception of dairy products.

LAB can catalyse reactions such as deamination, transamination and decarboxylation, and metabolism of their amino acids also contributes to the flavour. As an example, same strains of importance in bakery production convert glutamine to glutamate during sourdough fermentation, imparting taste to the bread (Gänzle *et al.* 2007). The expression of the arginine deaminase pathway in *Lactobacillus* spp. promotes higher production of ornithine, and thus enhances the formation of 2-acetyl pyrroline, which is responsible for the roasty note of wheat bread crumb (Gänzle *et al.* 2007).

The proteolytic activity is also important for other mechanisms; several antihypertensive peptides produced during milk fermentation have a strong activity against angiotensin I-converting enzyme (ACE), a dipeptidyl carboxypeptidase that plays a major role in the regulation of blood pressure within the renine angiotensin system (Riordan 2003), inducing blood pressure increase. *In vivo* studies evidenced a reduction of blood pressure after consumption of fermented milks (Pina and Roque 2008). Moreover, *in vitro* ACE inhibitory (ACEI) activity of different traditional fermented milks has been reported in the literature (Chaves-López *et al.* 2011). Thus, selection of microorganisms to be used in fermented products is gaining in importance, due to the inherent variations in their ability to produce bioactive peptides, particularly those with specific health claims (Ramchandran and Shan 2008).

Recently, LAB-induced proteolysis has been suggested as an efficient method for decreasing the toxicity of wheat and rye flours. Gliadins are among the most affected proteins by food fermentation and the extent of hydrolysis of monomeric gliadins (α-, β-, γ-, ω-gliadins) is strain specific (Di Cagno *et al.* 2002). Di Cagno *et al.* (2002) showed that selected proteolytic LAB could efficiently hydrolyse the 31-43 fragment of the toxic peptide A-gliadin. On the basis of these results, the same authors showed that selected LAB could completely hydrolyse the highly toxic 33-mer peptide over prolonged (12–24 h) and semi-liquid fermentation of a mixture of wheat and non-toxic flours. Breads produced with 12-hour sourdough fermentation retained acceptable quality and when consumed by coeliac individuals, no alterations in the baseline values could be observed. The selected LAB were also successfully used for the detoxification of other fermented foods (De Angelis *et al.* 2006).

A variety of fermented foods, especially protein-rich foods, may contain biogenic amines (BAs). During the fermentation process protein breakdown products, peptides and amino acids, used by spoilage and also by the fermentation microorganisms, represent precursors for BA formation (Bodmer *et al.* 1999). The consumption of foods with high concentrations of BAs can induce adverse

reactions such as nausea, headaches, rashes and changes in blood pressure (Ladero *et al.* 2010). Microorganisms suitable for food fermentation have been examined with regard to their potential to degrade histamine and tyramine (Fadda *et al.* 2001). A low potential for histamine and tyramine degradation among lactobacilli was noticed. In 35 well-known species with a practical function for the fermentation of dairy products and wine, Straub *et al.* (1995) observed a potential to form BAs only for a few strains.

Lipases and esterases

The lipolytic and esterolytic systems of LAB remain poorly characterized. Esterases from lactic acid bacteria may be involved in the development of fruity flavours in foods, and pregastric lipase and esterases are essential for the development of taste perception and typical flavour in Italian cheese. Microbial lipases and esterases may improve quality or accelerate the maturation of cheeses, cured bacon and fermented sausages. However, except for Parmigiano Reggiano, Pecorino and related Italian cheeses and blue cheeses, limited lipolysis occurs in cheese during ripening.

Lipolysis results in the formation of free fatty acids, which can be precursors of flavour compounds such as methylketones, secondary alcohols, esters and lactones. Generally, the role of LAB in lipolysis is less significant, but additional cultures, such as moulds in the case of surface-ripened cheeses, are often highly active in fat conversion. Flavours derived from the conversion of fat are particularly important in soft cheeses like Camembert and Roquefort (Smit *et al.* 2005).

Lipases are chemically defined as glycerol ester hydrolases (EC 3.1.1.3) that hydrolyse tri-, di- and monoglycerides present at an oil–water interface. Esterases (EC 3.1.1.6) hydrolyse esters in solution and may also hydrolyse tri- and especially di- and monoglycerides containing short-chain fatty acids (Medina *et al.* 2004). Esterases have been purified from several starter and LAB, including *Lc. lactis* (Chich *et al.* 1997), *Strep. thermophilus* (Liu *et al.* 2001) and *Lb. plantarum* (Gobbetti *et al.* 1997). All of them are serine enzymes that preferentially hydrolyse butyrate esters and are optimally active at pH 7. Some of them have no activity at pH 5.0; nevertheless, a very small amount of activity over a long time could result in significant hydrolysis of fat during cheese ripening. The major tributyrin esterase of *Lc. lactis* has been cloned, overexpressed and characterized (Fernandez *et al.* 2000).

Some probiotic strains of LAB can hydrolyse triglycerides, releasing most short- and medium-chain and essential fatty acids, which are valuable to today's health-conscious consumer. Medium-chain fatty acids (C6-C14), in particular, have become an accepted treatment for patients with malabsorption symptoms, a variety of metabolic disorders, cholesterol problems and infant malnutrition. These probiotic bacteria could alleviate lipase deficiency in the digestive tract during digestion (Medina *et al.* 2004).

Bacteriocins production

Bacteriocins are peptides produced by various bacteria that inhibit the growth of other bacteria. They could ensure the stability of fermented products, reduce microbial contamination during fermentation, inhibit the growth of moulds and prolong the microbiological spoilage time of baked goods (Juodeikiene *et al.* 2009).

In recent years, interest in starter/probiotic LAB has also grown substantially due to their potential usefulness as a natural substitute for food preservatives in the production of fermented foods with an enhanced shelf life and/or safety. *Lactobacillus* and *Lactococcus* include main strains with probiotic activity (Fuller 1989), producing bacteriocins (Altuntas *et al.* 2010).

The inhibitory host range and the molecular mass can be either large or small. Bacteriocins produced by LAB are divided into three classes: lantibiotics, small heat-stable non-lantibiotics and large heat-stable bacteriocins (Nes *et al.* 1996). Nisin, the best-known bacteriocin, is a lantibiotic that is produced by some strains of *Lc. lactis* and is used commercially in more than 50 countries as a food preservative to control the growth of spoilage and pathogenic bacteria.

Homofermentative *Pediococcus acidilactici* were isolated from spontaneous rye sourdoughs and characterized as producing pediocin Ac807 with antimicrobial activity against *Bacillus subtilis* (Narbutaite *et al.* 2008).

Exopolysaccharide production

Many food-grade microorganisms produce exopolysaccharides (EPS) (De Vuyst and Degeest 1999). EPS act as biothickeners and can be added to a variety of food products, where they serve as viscosifying, stabilizing, emulsifying or gelling agents (Tieking and Gänzle 2005).

They are divided into two classes: homopolysaccharides (HoPS), mainly glucan or fructans polymers; and heteropolysaccharides, with (ir)regular repeating units (De Vuyst and Degeest 1999). Heteropolysaccharide production is an important characteristic of many LAB involved in the production of fermented milks.

Lactic acid bacteria produce either homopolysaccharides, containing fructose or glucose residue, or heteropolysaccharides, composed of repeating units of several different sugars including glucose, galactose, fructose and rhamnose (De Vuyst *et al.* 2001). They may be involved in a wide variety of biological functions, including prevention of desiccation, protection from environmental stresses, adherence to different surfaces, pathogenesis and symbioses (Jolly *et al.* 2002). EPS-producing cultures have also been used to increase the moisture and improve the yield of low-fat Mozzarella cheese (Perry *et al.* 1998).

Glucan and fructans produced by fermenting LAB can strongly influence the quality of wheat bread in terms of bread volume and crumb firmness (Di Cagno *et al.* 2006). In particular, the production of EPS *in situ* is more effective than their addition (Brandt *et al.* 2003).

LAB can also produce gluco- or fructo-oligosaccharides (FOS), among which FOS, together with the fructan inulin, have been well described for their prebiotic effects (Biedrzycka and Bielecka 2004). In addition, the levan produced by *Lactobacillus sanfranciscensis* was proved to stimulate bifidobacterial growth *in vitro* (Dal Bello *et al.* 2001). In sourdough, *Lactobacillus reuteri, Lactobacillus acidophilus* and *Lb. sanfranciscensis* showed the ability to produce the prebiotic FOS 1-kestose (Tieking and Gänzle 2005).

Conclusion

The use of industrial starters has reduced the biodiversity and the organoleptic properties of fermented products. This phenomenon may be explained because the commercial availability of new, interesting starter cultures is very limited. Therefore, the selection of promising and wild strains from raw materials could be an interesting way forward. We can suggest at least three hot topics in selecting new LAB cultures: genome sequencing; interaction with natural microbiota; and functionality (Figure 1.2).

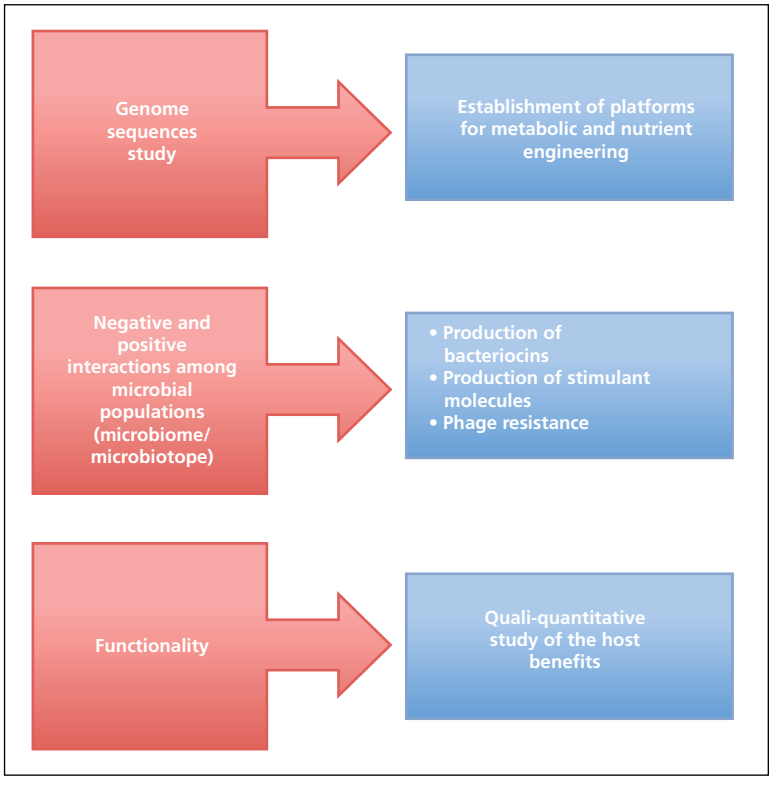

Figure 1.2 A new approach in the selection of microorganisms for innovative food purposes.

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CHAPTER 2
Yeasts as starter cultures

Pietro Buzzini, Simone Di Mauro and Benedetta Turchetti
Department of Agricultural, Food and Environmental Science, Industrial Yeasts Collection DBVPG, University of Perugia, Italy

Together with drying and salting, fermentation is one of the oldest ways to preserve perishable foods and beverages, dating back at least 6000 years (McGovern *et al.* 2004; Sicard and Legras 2011). Nowadays, the importance of fermented products for consumers is underlined by the broad variety of fermented foods and beverages marketed in both developing and industrialized countries, not only for their indisputable benefit of preservation and safety, but also for their highly appreciated sensory attributes. Microorganisms (and their enzymes) contribute to the improvement of some characteristic properties such as taste, aroma, visual appearance, texture, shelf-life and safety (Holzapfel 2002).

The need for inocula for starting the fermentative process was understood early and applied from time immemorial by keeping a sample (sometimes labelled a 'natural culture') from the previous production and using it as a starter. With the discovery of microorganisms, it became possible to improve fermented products by using well-characterized starter cultures. This became routine in the nineteenth century for producing wine, beer, vinegar and bread. In contrast, the dairy and meat industries began to use well-characterized starter cultures only about a century later (Hansen 2002; Holzapfel 2002).

A starter culture may be defined as a preparation containing a large number of (sometimes variable) technological microorganisms, which is inoculated to accelerate and guide a given fermentative process. A typical starter facilitates the control, improvement and predictability of fermentation only if it is well adapted to the substrate (Holzapfel 2002). Food technologists can currently choose either to purchase the starter culture in a ready-to-use and highly concentrated form or to propagate the culture in-house. The preference for one or other of the two methods is currently influenced by the type of fermented product to be obtained; the presence of in-house microbiological expertise and equipment facilities; and the economic impact. Overall, the highest level of safety and flexibility

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is achieved by using commercial starter cultures for direct inoculation. Such starters are usually supplied as dried (or freeze-dried), highly concentrated and active cultures in order to be easily used to inoculate the substrate (Hansen 2002).

Yeasts as starter cultures: General considerations

Although ancient peoples unknowingly used yeasts since antiquity for producing fermented foods and beverages, the awareness of the ability of these microorganisms to convert carbohydrates into ethanol and carbon dioxide (CO_2) dates back to experiments carried out by Louis Pasteur in 1860 (Sicard and Legras 2011). Yeasts are a group of eukaryotic unicellular organisms belonging to the kingdom of fungi and behave in nature as saprotrophs and degraders of organic macromolecules. They are currently used in fermentative processes, mainly because of their ability to utilize a broad variety of feedstock and to produce a number of valuable fermented foodstuffs (Tamang and Fleet 2009; Sicard and Legras 2011).

It has been suggested that the species belonging to the *Saccharomyces sensu stricto* complex (including *Saccharomyces cerevisiae*, commonly labelled 'baker's yeast') were the first example of organisms domesticated by humankind (Sicard and Legras 2011). Accordingly, most people associate yeasts almost exclusively with *Saccharomyces* species. In fact, it is not uncommon in some areas of microbiology, molecular biology and biotechnology to utilize the words 'yeast' and '*Saccharomyces*' as synonyms and to use the species *S. cerevisiae* as the primary model for studying the biology of eukaryotic organisms. This is in spite of evidence that this species represents only an infinitesimal part of the biodiversity existing in the yeast world (Buzzini and Vaughan-Martini 2006). It has been estimated that the number of yeast species so far described (approximately 1500) represents about 1% of the total predictable diversity (Boekhout 2005). Thus, there is enormous potential in studying new yeast species for their possible commercial use. Indeed, an increasing body of academic and industrial research has recently paid attention to several non-*Saccharomyces* species, mainly belonging to the genera *Candida*, *Debaryomyces*, *Kluyveromyces*, *Yarrowia*, *Pichia*, *Zygosaccharomyces* and so on, for possible exploitation as starter cultures for both food and non-food (industrial) technologies (Fleet 2006; Buzzini and Vaughan-Martini 2006; Romano *et al.* 2006).

Yeasts as starter cultures in winemaking

Starter cultures of *S. cerevisiae*

The first evidence of winemaking dates back to 5000 BCE in Mesopotamia and Greece (Bisson *et al.* 2002; Valamoti *et al.* 2007; Legras *et al.* 2007; Sicard and Legras 2011). Grape juice fermentation is a complex biochemical process wherein

yeasts play a fundamental role by converting carbohydrates into ethanol, CO_2 and several hundreds of secondary products, sometimes characterized by high volatility (Ciani *et al.* 2010). For many years, wines have been produced by spontaneous fermentation resulting from the competitive activities of a variety of contaminating indigenous yeasts (labelled 'wild yeasts') of the species *Hanseniaspora uvarum* (teleomorph state of *Kloeckera apiculata*), *Torulaspora delbrueckii*, *Pichia* spp., *Candida* spp. and so on. These indigenous yeasts usually dominate the mature grape yeast populations and, despite their inability to achieve complete fermentation, enhance the wine's aroma and flavour during the early stages of the winemaking process. The presence of alcohol-tolerant *S. cerevisiae* strains increases proportionally to the ethanol concentration during the mid to final phases of fermentation at the expense of the indigenous yeasts (Fleet 1999, 2003; Pretorius 2000; Holzapfel 2002; Calabretti *et al.* 2012). The number of indigenous species and their presence during the early phases of fermentation depends on several factors. This consequently determines much of the variation of wine quality from region to region, but also from one year to another (Pretorius 2000).

There is a general assumption that the inoculation of grape must with yeast starter cultures can overwhelm and suppress the growth of indigenous strains and dominate the fermentative process, thus improving the general quality of the wine. This theory has addressed the research of nearly a century into so-called super-selected yeast. Because of their dominance, strains of *S. cerevisiae* have been historically isolated, selected and commercialized for decades as starter cultures for winemaking. Many companies selling yeasts for the food and beverage industries were started in the last 50 years. Some of them conserve an in-house collection of strains, which are regularly subjected to periodic screening surveys for selecting specific starter cultures. Other companies, however, are merely 'sellers' of strains that have been isolated and selected by distinct microbiology laboratories or service culture collections. There is a list of major worldwide companies selling yeast starter cultures in Table 2.1.

The idea that inoculated fermentations can proceed more rapidly and predictably than their spontaneous counterparts is a universally recognized concept. Consequently, yeast starters are regularly utilized by many winemakers

Table 2.1 Major worldwide companies selling yeast starter cultures.

Name	Country	Website	Starters for
Angel Yeast	China	www.angelyeast.com	Beer, bakery products
Lallemand	Canada	www.lallemand.com	Wine, beer, bakery products
Lesaffre/Fermentis	France	www.lesaffre.com www.fermentis.com	Wine, beer, spirits, bakery products
White Labs	USA	www.whitelabs.com	Wine, beer, spirits

worldwide (Calabretti *et al.* 2012). However, molecular ecological studies have now reported that these assumptions are not necessarily correct. Indeed, the indigenous yeasts present in grape and must sometimes continue to contribute to fermentation (Fleet 1999). In order to monitor this phenomenon, a few molecular methods (i.e. mtDNA restriction analysis and comparison of chromosomal DNA profiles) have been proposed to check whether or not fermentation is successfully conducted by the inoculated starter yeasts (Torija *et al.* 2001).

In recent years wine technologists and winemakers have increasingly focused their interest on the use of autochthonous *S. cerevisiae* strains, with the aim of selecting starter cultures better adapted to a specific grape must in order to try to reflect the biodiversity of a given region. This approach is supported by the hypothesis that specific native strains can be associated with a given territory, or even with a particular winery (Torija *et al.* 2001; Lopes *et al.* 2002; Capece *et al.* 2010; Settanni *et al.* 2012). The recent discovery that an overabundance of *S. cerevisiae* living cells is present on the surfaces of wineries has made available a large reservoir of yeast diversity to be used as a source of locally selected starters for winemaking. A few studies have postulated that any winery potentially hosts a local, resident population of *S. cerevisiae* strains, which are technologically optimized for winemaking and adapted to produce a set of peculiar compounds possibly involved in the formation of (sometimes individual) aromas. The logical consequence is that any winery may potentially contain its own 'super-selected' starter producing personalized sensory characteristics (Martini 2003). This approach has also proven to be very effective for selecting commercial 'winery-specific' strains, which are ideal for the production of typical regional wines. Accordingly, a number of researchers have recently characterized *S. cerevisiae* cultures isolated from worldwide wine cellars (Domizio *et al.* 2007; Lopes *et al.* 2007; Valero *et al.* 2007; Capece *et al.* 2010; Settanni *et al.* 2012; Tristezza *et al.* 2012; Mazzei *et al.* 2013; Elmacı *et al.* 2014).

Conventionally, the selection of *S. cerevisiae* starters for winemaking has mainly been approached by using two oenological traits (Martini 2003): primary characteristics, defined as those strictly associated with the formation of ethanol by fermentation; and secondary qualities, related to the production of compounds affecting other parameters, namely the body of a wine (e.g. glycerol), the higher alcohols complex (bouquet) and the appearance of either desirable flavours or undesirable off-flavours. Large-scale screening surveys are still ongoing worldwide particularly aimed at finding the optimal starter for specific wines (often of great value) for both traditional and modern cellars. Wines obtained from different starters have been evaluated for their chemical composition and sensory characteristics (Pretorius 2000; Pretorius *et al.* 2003; Dequin 2001; Bisson 2004; Borneman *et al.* 2007). Advances have been made in yeast fermentation vigour and complete utilization of carbohydrates, and in wine processing (including clarification) and enhanced formation of desired aromas, which is a complex and important aspect of wine quality because the physiology and

neurobiology of human olfaction and the assessment of the desired sensory properties have significant impacts on the desirability and economics of wine (Bisson *et al.* 2002). The decrease of possible off-flavours (to enhance the organoleptic qualities of wines) has also been targeted as an additional selection criterion (Pretorius *et al.* 2003; Bisson 2004; Borneman *et al.* 2007).

A number of additional challenges have been addressed in recent years (Moreno-Arribas and Polo 2005). Among them the possible use of starter cultures at low temperatures is worthy of note. It is well known that fermentative processes performed at temperatures below 15 °C lead to more aromatic and paler wines (Bauer and Pretorius 2000; Ribéreau-Gayon 2006). Low temperatures increase the duration of alcoholic fermentation, decrease the rate of yeast growth and modify the ecology of wine fermentation (Torija *et al.* 2003). The pre-adaptation of starter cultures of *S. cerevisiae* to cold conditions could improve fermentation performance, although this improvement is strain dependent. Low-temperature fermentations also determine the reduction of acetic acid and fusel alcohol production and increase the concentrations of glycerol (Llauradó *et al.* 2005). The technological and sensory characteristics of *S. cerevisiae* strains grown at low temperatures have recently been reviewed (Kanellaki *et al.* 2014).

The production of wines with a reduced concentration of ethanol and chemical preservatives represents an additional target for many wine cellars selling their product in developed nations, due to the growing consumer demand for wines containing lower levels of ethanol and chemical additives (labelled 'organic' wines). Both purposes have been pursued by using techniques of DNA mutation or recombination in starter cultures of *S. cerevisiae* (Johnson and Echavarri-Erasun 2011). The first target is related to the increased interest in healthy lifestyles linked to lowering excessive alcohol consumption, as well as concerns related to wine quality, because high alcohol concentrations exert a masking effect on the flavours and aromas of wine (Guth and Sies 2002). In this context, the use of low ethanol–producing yeasts may be considered a cheap opportunity (Rossouw *et al.* 2013). Genetic manipulation of *S. cerevisiae* strains for reducing their ability to accumulate ethanol has been supported by current literature on the regulatory mechanisms of yeast fermentative metabolism (Rossignol *et al.* 2003; Trabalzini *et al.* 2003; Varela *et al.* 2005; Howell *et al.* 2006; Zuzuarregui *et al.* 2006; Marks *et al.* 2008; Rossouw and Bauer 2009). Glycolytic genes are slowly down-regulated as fermentation progresses, with only a few exceptions where isoforms of the same protein are differentially expressed (Varela *et al.* 2005; Marks *et al.* 2008). Under glucose-repressed fermentative conditions, genes encoding the tricarboxylic acid cycle appear to be underexpressed during fermentative metabolism. Additional investigation concerning metabolic C fluxes under simulated fermentation conditions drew attention to discrepancies between these fluxes and the corresponding gene expression patterns (Varela *et al.* 2005). Malherbe *et al.* (2003) expressed the *Aspergillus niger*

gene encoding a glucose oxidase in *S. cerevisiae* in order to obtain lower alcohol production and inhibition of spoilage bacteria. *S. cerevisiae* transformants exhibited slightly reduced alcohol production, probably as a consequence of the parallel production of gluconic acid from glucose by glucose oxidase. In contrast, Rossouw *et al.* (2013) screened a set of *S. cerevisiae* mutants exhibiting deletion of genes encoding enzymes involved in central carbohydrate metabolism (i.e. trehalose biosynthesis, central glycolysis, oxidative pentose phosphate pathway and tricarboxylic acid cycle) for their impact on ethanol yields. A TPS1 gene (encoding trehalose-6-phosphate synthase) was selected as a putative candidate to alter flux to ethanol during alcoholic fermentation. The expression of the TPS1 gene was slightly up-regulated, resulting in a decrease in ethanol production and an increase in trehalose biosynthesis. Additional advances in the selection of wine yeasts were realized through DNA technology in *S. cerevisiae* strains to improve a few stress properties, including osmotolerance and ethanol resistance (Johnson and Echavarri-Erasun 2011).

Mixed starters for co-fermentations

It is generally assumed that wine produced by using pure cultures of *S. cerevisiae* can sometimes lack the complexity of taste and sensory characteristics produced by indigenous yeasts in spontaneous fermentative processes. Since the early 2000s this has stimulated the 'rediscovery' of indigenous non-*Saccharomyces* yeasts (as co-starters in association with *S. cerevisiae*) for producing wines characterized by a high aroma content (Romano *et al.* 2003; Cheraiti *et al.* 2005; Calabretti *et al.* 2012). This fascinating topic has attracted the work of a growing number of wine microbiologists in order to study the impact of non-*Saccharomyces* yeasts on the composition, sensory properties and final flavours of wine. Indeed, it is known that the yeast ecology of the fermentative process is more complex than previously thought, and that some non-*Saccharomyces* yeasts can play a relevant role in the fermentation dynamics, metabolic impact and aroma complexity of the final product (Swiegers and Pretorius 2005; Domizio *et al.* 2007; Renouf *et al.* 2007; Fleet 2008; Ciani *et al.* 2010; Calabretti *et al.* 2012). The volatile compounds responsible for varietal aroma in wine are mainly terpenes, wherein free forms of monoterpenes are the most important group because of their high volatility. The glycosylated terpenes can be hydrolysed (by β-glycosidases produced by some non-*Saccharomyces* strains) to the corresponding free forms during the early phases of winemaking (Calabretti *et al.* 2012). Because of their growing importance, most studies recently proposed the use of mixed or sequential inoculation of *S. cerevisiae* and non-*Saccharomyces* strains as a feasible way for improving the complexity and enhancing some specific traits of wines (Romano *et al.* 2003; Clemente-Jimenez *et al.* 2005; Moreira *et al.* 2005, 2008; Rodríguez *et al.* 2010; Ciani *et al.* 2010; Clavijo *et al.* 2011; De Benedictis *et al.* 2011; Viana *et al.* 2011; Calabretti *et al.* 2012; Hong and Park 2013; del Mónaco *et al.* 2014).

Yeasts as starter cultures in brewing

Beer is one of the most widely consumed alcoholic beverages in the world. It was first mentioned in ancient Mesopotamian literature, which dates back to the seventh century BCE (Sicard and Legras 2011). Some studies revealed that the species *S. cerevisiae* (currently used to produce ale beer) includes strains displaying a multiple ploidy and consequently great genome variability (Pedersen 1986). The complete sequencing of the genome of *Saccharomyces pastorianus* (synonym *Saccharomyces carlsbergensis*) W34/70 (a strain largely used for European lager beer) has been deciphered, revealing that it is an allopolyploid interspecies hybrid between *S. cerevisiae* and *Saccharomyces bayanus* (Nakao *et al.* 2009). In contrast, it was recently proposed that other strains of *S. pastorianus* could be originated from an interspecific cross between *S. cerevisiae* and the wild species *Saccharomyces eubayanus*. The draft genome sequence of *S. eubayanus* appears to be 99.5% identical to the non-*S. cerevisiae* portion of the *S. pastorianus* genome sequence. This suggests specific changes in carbohydrate and sulfite metabolism, which were crucial for domestication in the lager brewing environment (Libkind *et al.* 2011).

Many strains of the two closely related species *S. cerevisiae* and *S. pastorianus* have been selected in the last 15 years and proposed for brewing. Targeted properties for starter selection included high-fermentation performances in normal and high-gravity worts; optimal formation of organic acids, volatile compounds, glycerol and other molecules important for beer quality; enhanced flocculation after primary fermentation to favour beer clarification; cell viability as a function of time, temperature and ethanol concentration during storage; and use of a continuous fermentative process utilizing immobilized *S. cerevisiae* cells (Dequin 2001; Verbelen *et al.* 2006; Willaert and Nedovic 2006; Blieck *et al.* 2007; Bleoanca *et al.* 2013).

During the past few decades a number of technological factors have been targeted for the genetic improvement of brewer's yeasts (Bamforth 2000; Dequin 2001). The construction of yeast strains able to secrete heterologous β-glucanases (from *Aspergillus* spp.) to decrease viscosity and to promote more efficient filtration has also been recently proposed (Johnson and Echavarri-Erasun 2011). A strong emphasis has also been devoted to the ability of brewer's yeasts to utilize dextrins, which represent about 25% of malt wort carbohydrates and have a high caloric impact on low-alcohol beers (Johnson and Echavarri-Erasun 2011).

The excess of formation of diacetyl and other vicinal ketones can be considered negative to the savoury properties of beers. Bacterial genes encoding the production of α-acetolactate decarboxylase to enhance the amount of acetoin and to decrease diacetyl have been expressed in brewer's yeasts (Bamforth and Kanauchi 2004). In addition, as altered concentrations of sulfur compounds, as well as other off-flavours (e.g. staling, cardboard flavour attributed to (*E*)-2-nonenal, undesirable aromas derived from lipid oxidation etc.) can be considered nasty

by consumers, yeast strains have been developed exhibiting a modified sulfur metabolism and producing superior levels of sulfite with enhanced antioxidant and antibacterial properties (Vanderhaegen *et al.* 2006; Johnson and Echavarri-Erasun 2011).

Yeasts as starter cultures in bakery products

Historically, fermented cereals have played a significant role in human nutrition in all parts of the world where cereals grow (Hammes *et al.* 2005). The first report on bread making dates back to ancient Egypt (Sicard and Legras 2011). Generally, baker's yeast (*S. cerevisiae* is the most common species in bread making) is required to have several technological characteristics, namely a high carbohydrate fermentation rate, sometimes cryotolerance, and a high leavening ability to ensure high-quality baking products (Rollini *et al.* 2007; Wongkhalaung and Boonyaratanakornkit 2007; Giannone *et al.* 2010; Cukier de Aquino *et al.* 2012). Nowadays, the bakery industry offers several commercial starter cultures, the choice of which depends on the type of bread-making technology. Compressed yeast is the form most widely used, but dry yeasts are in successful expansion because they are easy to use, even if their production is time-consuming and they require additional energy costs due to both drying and packaging processes (Papapostolou *et al.* 2012).

Targeted properties for selecting *S. cerevisiae* starters for bakery products include tolerance of high levels of sucrose (doughs can contain up to 30% sucrose, which exerts severe osmotic stress on yeast cells); tolerance of freezing–thawing stress; rapid utilization of maltose; and production of high levels of CO_2 (Verstrepen *et al.* 2006). Like wine and beer yeasts, the use of DNA technology has allowed significant advances in the construction of improved starters for the bakery industry (Johnson and Echavarri-Erasun 2011). *S. cerevisiae* strains exhibiting high sucrose tolerance and rapid utilization of maltose have been proposed for commercial use (Higgins *et al.* 2001). Genes encoding sucrose tolerance and maltose utilization have been studied in depth by using a functional genomics approach. The expression of genes involved in the accumulation and metabolism of glycerol and trehalose, and in resistance to osmotic stress, was demonstrated to be higher in sucrose-tolerant yeasts (Tanaka-Tsuno *et al.* 2007).

The freezing–thawing survival of yeasts is an attribute that would benefit the production and quality of frozen doughs (Rosell and Gomez 2007). Accordingly, cryoresistant *S. cerevisiae* and *T. delbrueckii* strains have been developed (Tanghe *et al.* 2003; Hernández-López *et al.* 2007). The ability of *S. cerevisiae* strains to utilize melibiose is particularly important because raffinose (a prominent component of molasses, an ingredient currently used in some bakery products) is hydrolysed by yeast invertase to fructose and melibiose. The expression of heterologous genes (from *S. pastorianus*) encoding α-galactosidase in

S. cerevisiae increased biomass without alteration of growth rate in model bakery fermentations (Dequin 2001). Another desired property in bread making is a rapid fermentation rate, sometimes related to maltose concentration. Dough amylases release maltose from starch, but many strains of *S. cerevisiae* utilize maltose poorly, primarily due to repression of maltose utilization by other sugars through catabolite repression. The possibility of overcoming this bottleneck could allow improvements in baking productivity (Johnson and Echavarri-Erasun 2011).

Sourdoughs, consisting of a mixed culture of yeasts and lactic acid bacteria, are alternative starter cultures frequently used worldwide in bakery foodstuffs. They have the advantage of improving the nutritional value, sensory qualities and texture of bread, enabling the baking of doughs for rye bread production and increasing the shelf-life of bread (Hammes *et al.* 2005). The yeast diversity of sourdoughs consists of specifically adapted strains, namely *S. cerevisiae* and *Kazachstania exigua* (synonym *Saccharomyces exiguus*; Hammes and Gänzle 1998). Although sourdoughs have been studied in depth in the past few years, research on their microbiological composition underwent a renaissance in the early 2000s, leading to some studies dedicated to characterizing their yeast diversity (Pepe *et al.* 2003; Edema and Sanni 2008; Vogelmann *et al.* 2009; Moroni *et al.* 2010).

Yeasts as starter cultures in dairy products

Cheese

Microbial communities occurring in cheeses are initially dominated by lactic acid bacteria, while yeasts are believed to have a significant role in ripening and flavour development (Viljoen 2001; Hui *et al.* 2004). Fundamental studies on yeast diversity in cheese date back to the early 1990s. Yeasts' wide occurrence in cheese making may be attributed to their ability to tolerate high salt (NaCl) concentrations, low pH and water activity, as well as to their aptitude to grow at low temperatures and to assimilate lactose and lactic acid. In addition, their high proteolysis and lipolysis are considered crucial for releasing soluble amino acids and free fatty acids (Wyder and Puhan 1999).

The use of yeasts of the species *Debaryomyces hansenii* and *Yarrowia lipolytica* as starter cultures for cheese making has been proposed since the 1990s due to their positive impact in cheese ripening, NaCl resistance, ability to grow vigorously in cheese systems, as well as compatibility with lactic acid bacteria in mixed starter cultures (Wyder and Puhan 1999; van den Tempel and Jakobsen 2000; van den Tempel and Nielsen 2000; Guerzoni *et al.* 2001; Suzzi *et al.* 2001). Hence, both *D. hansenii* and *Y. lipolytica* (and occasionally other yeast species) have been proposed as co-starters with lactic acid bacteria, micrococci and/or filamentous fungi in cheese making worldwide (Wyder and Puhan 1999; van den Tempel

and Nielsen 2000; Guerzoni *et al.* 2001; Hansen and Jakobsen 2001; Hansen *et al.* 2001; Psomas *et al.* 2001; Suzzi *et al.* 2001; Ferreira and Viljoen 2003; Żarowska *et al.* 2004; Goerges *et al.* 2008; Papapostolou *et al.* 2012; Gkatzionis *et al.* 2014).

Whey

Whey is the pale yellow residual liquid obtained after the flocculation and removal of milk casein during cheese making. This by-product represents approximately 85% of the milk volume and retains 55% of milk nutrients, including lactose, whey proteins, lipids and mineral salts (Dragone *et al.* 2009). Very few yeast species are lactose positive, but most strains are able to utilize the galactose, lactic acid or even citric acid that are present in whey, depending on the cheese-making technology. Whey represents a global environmental problem because of the high volumes produced and high BOD and COD values (Smithers 2008; Guimarães *et al.* 2010). Accordingly, several methods have been proposed for its economic exploitation, among them the production of ethanol (as biofuel) by lactose-positive genetically engineered *S. cerevisiae* strains (Domingues *et al.* 2001, 2010; Guimarães *et al.* 2008).

Fermented milk

Many yeast species have been isolated from commercial fermented milk products, in particular *Kluyveromyces marxianus*, *D. hansenii*, *Y. lipolytica* and *Rhodotorula mucilaginosa* (Rohm *et al.* 1990; Jordano *et al.* 1991; McKay 1992). Kefir is yeast-containing fermented milk traditionally produced in-house in Europe and Asia. Although its microbiological composition has been well characterized, considerable variations have been apparently observed among different worldwide cultures. Wyder (1998) found 23 yeast species, in particular *K. marxianus* and *S. cerevisiae*. More recently, some studies have reported the presence of both culturable and non-culturable yeast diversity in kefir grains collected worldwide, predominantly strains belonging to the genera *Candida*, *Kazachatania*, *Kluyveromyces*, *Pichia*, *Saccharomyces* and *Zygosaccharomyces* (Garbers *et al.* 2004; Witthuhn *et al.* 2004; Jianzhong *et al.* 2009; Magalhães *et al.* 2011; Kök Taş *et al.* 2012; Gao *et al.* 2012, 2013; Leite *et al.* 2012; Miguel *et al.* 2013; Diosma *et al.* 2014).

Yeasts as starter cultures in fermented meat products

The dynamic of microbial communities occurring in fermented meats is similar to that observed in cheeses; these products are primarily colonized by lactic acid bacteria, while yeasts often (together with micrococci and filamentous fungi) play a secondary role in ripening and flavour development (Hui *et al.* 2004). Some studies have shown the impact of the extracellular proteolytic and lipolytic enzymes produced by yeasts on the development of the characteristic tastes and flavours of fermented meats (Durá *et al.* 2004; Flores *et al.* 2004;

Martín *et al.* 2006). Most of the sensory properties are attributed to the hydrolysis of lipids and proteins and to the release of small peptides, amino acids and free fatty acids during the ripening process (Patrignani *et al.* 2007; Andrade *et al.* 2009).

A number of recent studies have characterized yeast communities occurring in fermented meats in order to select strains of *D. hansenii* and *Y. lipolytica* (and occasionally *Candida* spp.) as possible commercial starters, occasionally for mixed fermentation (Coppola *et al.* 2000; Olesen and Stahnke 2000; Bozkurt and Erkmen 2002; Baruzzi *et al.* 2006; Martín *et al.* 2006; Iucci *et al.* 2007; Patrignani *et al.* 2007; Sánchez-Molinero and Arnau 2008; Andrade *et al.* 2009; Purriños *et al.* 2013).

Yeasts as starter cultures in miscellaneous fermented foods and beverages

Fermented olives

It is generally accepted that yeasts can produce compounds exhibiting important organoleptic attributes improving the quality of fermented olives, especially ethanol, glycerol, higher alcohols, organic acids, acetaldehyde, esters and other volatile compounds, which may play an important role in flavour generation during the process (Montaño *et al.* 2003; Sánchez *et al.* 2000; Arroyo-Lopez *et al.* 2008, 2012). The lipolytic activity exhibited by some strains could also improve the volatile profile of these foodstuffs by increasing their free fatty acid content. The biodegradation of polyphenols catalyzed by specific β-glucosidases synthesized by yeasts is another interesting technological feature that could reduce the large quantities of olive wastewater produced during the lye treatment for fruit debittering (Hernández *et al.* 2007; Rodríguez-Gómez *et al.* 2010, 2012).

Against this background, the selection of yeast starters (in particular species of the genera *Candida*, *Kluyveromyces*, *Debaryomyces* and *Saccharomyces*) for olive fermentation is considered a key step for improving the process both in the laboratory and at an industrial scale (Hernandez *et al.* 2007; Arroyo-Lopez *et al.* 2012; Bevilacqua *et al.* 2012, 2013; Corsetti *et al.* 2012; Pistarino *et al.* 2013).

Cocoa

The first step of the chocolate-making process involves both pectinolysis and fermentation of cocoa beans. At present, cocoa is almost exclusively transformed by using spontaneous fermentations driven by natural microbial consortia, which include yeasts (in particular *S. cerevisiae*), lactic acid bacteria and acetic acid bacteria (Boekhout and Samson 2005; de Melo Pereira *et al.* 2012). Yeast metabolism in cocoa fermentation (conversion of pulp sugars into ethanol, release of pulp-degrading pectinases and development of chocolate aroma) has recently been elucidated (Nielsen *et al.* 2007; Ho *et al.* 2014). Several studies have

investigated yeast diversity in both spontaneous and controlled cocoa fermentations: species of the genera *Candida*, *Hanseniaspora*, *Hyphopichia*, *Kodamaea*, *Pichia*, *Meyerozyma*, *Kluyveromyces*, *Saccharomyces*, *Trichosporon* and *Yamadazyma* have been found (Ardhana and Fleet 2003; Boekhout and Samson 2005; Jespersen *et al.* 2005; Nielsen *et al.* 2007; Daniel *et al.* 2009; Papalexandratou and De Vuyst 2011; de Melo Pereira *et al.* 2012; Lefeber *et al.* 2012; Crafack *et al.* 2013; Ho *et al.* 2014).

Coffee

Coffee is one of the most globally appreciated non-alcoholic drinks. Coffee fermentation is a spontaneous process characterized by the presence of different microorganisms, including bacteria, filamentous fungi and yeasts that produce enzymes, namely polygalacturonases and pectin-lyases, which are necessary to depolymerize and hydrolyze the pectins present in the mucilage. Among these, some pectinolytic yeasts of the species *K. marxianus* (syn. *Saccharomyces marxianus*), *Pichia kluyveri*, *S. bayanus*, *S. cerevisiae*, *Schizosaccharomyces* sp. and *Wickerhamomyces anomalus* (syn. *Pichia anomala*) have been proposed as starters for the fermentation of coffee cherries (Kashyap *et al.* 2001; Jayani *et al.* 2005; Masoud and Jespersen 2006; Silva *et al.* 2013).

Fermented fruit and vegetables

The combined effect of lactose intolerance, high cholesterol content, ergenic milk proteins and aspiration for vegetarian alternatives is quickly pushing consumer demand towards the replacement of dairy foodstuffs with products obtained by the fermentation of fruits and vegetables (Heenan *et al.* 2005; Granato *et al.* 2010; Rivera-Espinoza and Gallardo-Navarro 2010). There are a wide variety of traditional non-dairy fermented beverages produced around the world, which represent a huge economic potential for the global food industry. Many of them are non-alcoholic beverages produced using legumes (e.g. soy milk) or cereals as raw materials (Prado *et al.* 2008). Nevertheless, fruit juices and vegetable-based fermented products have also been proposed. There have been studies of the impact of the use of mixed starter cultures (including *S. cerevisiae*, *Pichia fermentans* and lactic acid bacteria) on their organoleptic, sensory and functional attributes (Rodríguez-Lerma *et al.* 2011; do Amaral Santos *et al.* 2014).

Yeasts as starter cultures in worldwide ethnic fermented foods and beverages

Fermented foods and beverages from Africa and Asia

Some studies on the microbial diversity occurring in some African naturally fermented milk have reported the considerable presence of yeasts as a relevant part of natural microbial consortia (Pedersen *et al.* 2012). Contamination from

the environment and the equipment associated with fermentation could be assumed to work as natural inoculum of yeasts in these foods and beverages (Beukes *et al.* 2001; Narvhusa and Gadaga 2003). A number of studies have been carried out to characterize yeast diversity in ethnic fermented foods and beverages in order to select suitable starter cultures (or co-starters, in association with lactic acid bacteria) for improving safety and quality, sensory features and, sometimes, probiotic properties (Annan *et al.* 2003; Nyanga *et al.* 2007; Shetty *et al.* 2007; Vieira-Dalodé *et al.* 2007; Padonou *et al.* 2010; Pedersen *et al.* 2012; Greppi *et al.* 2013). Salient information on the main yeast species found in some African fermented foods and beverages is reported in Table 2.2. In Oriental countries some 'natural starters' (sometimes in the form of dry powders or hard balls made from starchy cereals) are frequently used to inoculate raw materials. There have been studies of yeast diversity in Asian fermented foods and beverages (Tsuyoshi *et al.* 2005; Sridevi *et al.* 2010; Wu *et al.* 2011). Salient information on the main yeast species found in some Asian fermented foods and beverages is listed in Table 2.3.

Fermented foods and beverages from South America

Yeast diversity in South American fermented foods and beverages has been studied recently, in particular in the so-called cachaça, a rum-like spirit produced from sugar cane (Gomes *et al.* 2007; Oliveira *et al.* 2008; Campos *et al.* 2010; Gonçalves de Sousa *et al.* 2012). The spontaneous process of fermenting sugar cane juice usually uses natural microbial starter cultures (containing yeasts that are not well defined; Schwan *et al.* 2001). The isolation of indigenous strains from the local production area and their selection and use as starters could ensure the adequate control of alcoholic fermentation and preserve some positive organoleptic contributions (Gomes *et al.* 2007; Oliveira *et al.* 2008; Campos *et al.* 2010; Gonçalves de Sousa *et al.* 2012). Indeed, some modern industrial processes recommend the addition of starter cultures of *S. cerevisiae* (in the form of active dry yeasts) to speed up the fermentative process, increase the levels of the desired metabolites and prevent the production of deleterious components by microbial contamination (Campos *et al.* 2010).

Yeasts as biocontrol agents in foods and beverages

Spoilage of food and beverages is a serious problem for industry: it can make products unacceptable to consumers and can cause economic losses and potentially severe health hazards. Many spoilage yeasts can grow when good manufacturing practices are not correctly employed (Viljoen *et al.* 2003; Stratford 2006). Food-grade antimicrobial compounds (e.g. sorbic and benzoic acids) are routinely used for prolonging shelf life and the preservation of food quality by inhibiting spoilage microorganisms (Battey *et al.* 2002; Papadimitriou *et al.* 2007).