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COMMISSION ON GENETIC RESOURCES FOR FOOD AND AGRICULTURE

STATUS AND TRENDS OF THE CONSERVATION AND SUSTAINABLE USE OF MICRO-ORGANISMS IN FOOD PROCESSES

By

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This document has been prepared at the request of the Secretariat of the FAO Commission on Genetic Resources for Food and Agriculture, and in close collaboration with the FAO Rural Infrastructure and Agro-Industries Division, to facilitate the Commission's discussions when it will review key issues in micro-organisms and invertebrates at its Fourteenth Regular Session.

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Acronyms and Abbreviations

2DE: Two dimensional gel electrophoresis
Ac.: *Acetobacter*
ACM: Asian Consortium for Microorganisms
AFB_1: Aflatoxin B_1
ARC: Agricultural Research Council, Plant Protection Research Institute (Rep. of S. Africa)
ASCRS: Australian Starter Culture Research Centre
Asp.: *Aspergillus*
ATCC: American Type Culture Collection
a_w : water activity
B.: *Bacillus*
Bbm.: *Bifidobacterium*
BCCM: Belgian Coordinated Collections of Microorganisms (www.bccm.belspo.be)
BIOHAZ: EFSA Panel on Biological Hazards
BMBF: German Federal Ministry of Research and Education
BOD: Biological (or Biochemical) Oxygen Demand
BRC: Biological Resource Centre
Bro.: *Brochothrix*
C.: *Clostridium*
CABRI: Common Access to Biological Resources and Information (www.cabri.org)
Cand.: *Candida*
CAGR: Compound Annual Growth Rate
Cb.: *Carnobacterium*
CBD: Convention on Biological Diversity
CCT: Fundacao Tropical Andre Tosello, Colecao de Culturas Tropical (Brazil)
CFU: Colony forming units
CGIAR: Consultative Group on International Agriculture Research
CODEX: Codex Alimentarius
CRS: Catholic Relief Services
DANIDA: Danish International Development Agency
Deb.: *Debaryomyces:*
DMSO Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
E.: *Escherichia*
EBRCN: European Biological Resource Centres Network (www.ebrcn.net)
EEC: European Economic Community
ECCO: European Culture Collection Organization (www.eccosite.org)
EFARD: European Forum on Agricultural Research for Development
EFFCA European Food and Feed Cultures Association
EFSA: European Food Safety Agency
Eh: Redox potential
ELISA: Enzyme-linked imumunoabsorbent assay
EMbaRC: European Consortium of Microbial Resources Centres (www.embarc.eu)
EMC: Enzyme Modified Cheeces
Ent.: *Enterococcus*
ESFRI: European Strategy Forum for Research Infrastructure

EU: European Union
F&B: Food and Beverages
FAM: Food Aid Management
FAO: Food and Agriculture Organization
FARA: Forum for Agricultural Research in Africa
FDA Food and Drug Administration
FHI: Food for the Hungry
FIAN: Food First Information and Action Network International
FOSHU: Foods for Specified Health Use
GBRCN: Global Biological Resource Centre Network (www.gbrcn.org)
GC: Gas chromatography
GC/MS: Gas chromatography/mass spectrometry
Ge.: *Geotrichum*
GHP: Good hygienic practice
GI: Gastrointestinal
GIT: Gastro-intestinal tract
GIZ: Deutsche Gesellschaft für Internationale Zusammenarbeit
GLP: Good Laboratory Practice
GM: Genetically modified
GMM: Genetically modified microorganisms
GMO: Genetically Modified Organism
GMP: Good Manufacturing Practice
GPMs: Genome-probing microarrays
GR: Genetic resources
GRAS: Generally Recognised as Safe
GtZ: Deutsche Gesellschaft für Technische Zusammenarbeit (GIZ since 2011)
ICARDA: International Center for Agricultural Research in the Dry Areas
ICRO: International Cell Research Organisation
HACCP: Hazard Analysis and Critical Control Points
HMP: Human Microbiome Project
HPLC: High performance liquid chromatography
ICFMH: International Committee on Food Microbiology and Hygiene (http://icfmh.org)
IMI: International Mycological Institute, Department of Mycology (UK)
INBio: Instituto Nacional de Biodiversidad, Department of Bioprospection (Costa Rica)
INCO-DC: International Cooperation with Developing Countries of the EU (1994-1998)
IPM: Industrial Platform for Microbiology (Belgium)
IPR: Intellectual property rights
ISO: International Standards Organization
IUBS: International Union of Biological Sciences
IUCN: World Conservation Union, Environmental Law Centre (Germany)
IUFoST: International Union of Food Science and Technology (http://iufost.org/about-iufost)
IUMS: International Union of Microbiological Societies (http://www.iums.org)
JECFA: Joint FAO/WHO Expert Committee on Food Additives
JFCC: Japanese Federation for Culture Collections
Kl.: *Kluyveromyces*
LAB: Lactic acid bacteria
Lact.: *Lactococcus*
Lb.: *Lactobacillus*

Leuc.: *Leuconostoc*
Li.: *Listeria*
LPFP: Lightly Preserved Fish Products
MALDI-TOF: Matrix-assisted laser desorption/ionization – Time of flight
MAP: Modified Atmosphere Packaging
MCA: Metabolic control analysis
ME: Metabolic engineering
MFA: Metabolic flux analysis
MIRCENs: Microbial Resource Centres
MIRRI: Microbial Resource Research Infrastructure (www.mirri.org)
MOSAICC: Microorganisms Sustainable Use and Access Regulation International Code of Conduct
MRC: Microbial Resource Center
MS: Mass spectrometry
MSDN: Microbial Strain Data Network
Mu.: *Mucor*
NEPAD: The New Partnership for Africa's Development
NMR: Nuclear magnetic resonance
NZDRI: New Zealand Dairy Research Institute
OECD: Organisation for Economic Co-ordination and Development
ORFs: Open reading frames
OTA: Ochratoxin A
OTU: Operational Taxonomic Unit
P: *Pichia*
PAB Propionic Acid Bacteria
PAEPARD: Platform for African-European Partnership on Agricultural Research for Development
PASSCLAIM: EU-project Process fort he Assessment of Scientific Support for Claims on Foods
PCR: Polymerase chain reaction
Ped.: *Pediococcus*
Pen.: *Penicillium*
Pi.: *Pichia*
Ps.: *Pseudomonas*
QHC: Qualified Health Claims
QPS: Qualified Presumption of Safety (EU) (adopted 2005: EFSA-Q-2004-021)
R: *Rhodococcus*
RAPD: Randomly amplified polymorphic DNA
Rhiz.: *Rhizopus*
RI: Research Infrastructure
RNA: Ribonucleic acid
RT-qPCR: Real time quantitative polymerase chain reaction
S.: *Salmonella*
Sacch. : *Saccharomyces*
Sm: *Saccharomycopsis*
SOM: Self-Organising Map
SSA: Significant Scientific Aggreement
Staph.: *Staphylococcus*
Strep.: *Streptococcus*
T.: *Tetragenococcus*

TLC:	Thinlayer-chromatography
To.:	*Torula*
Tor.:	*Torulopsis*
UICC: (Indonesia)	Universitas Indonesia Culture Collection, Laboratory of Microbiology
UK:	United Kingdom
UKFCC:	United Kingdom Federation for Culture Collections
UKNCC:	United Kingdom National Culture Collection (www.ukncc.co.uk)
UNEP:	United Nations Environmental Programme
UNESCO:	United Nations Educational, Scientific and Cultural Organization
UNISPAR:	University-Industry Science Partnership (under UNESCO)
US:	United States
USD:	United States Dollar
USFCC:	United States Federation for Culture Collections
W.:	*Weissella*
WAITRO:	World Association of Industrial and Technological Research Organizations
WDCM:	World Data Center for Microorganisms (www.wdcm.org)
WFCC:	World Federation of Culture Collections (www.wfcc.info)
WHO:	World Health Organization
WIPO:	World Intellectual Property Organisation

EXECUTIVE SUMMARY

The use of microorganisms in food processing constitutes a major part of Food Biotechnology by which relatively bulky, perishable and frequently inedible raw materials are converted into safe, shelf-stable and palatable foods or beverages. While food fermentations contribute an important (but not necessarily vital) part to the diet in industrialised countries, they play an essential role in the nutrition of developing countries. The value and benefits of fermented foods are realised in our time perhaps more than ever before. Reasons for an increasing awareness are multi-fold, and may differ between industrialised and developing countries. Basic understanding of mechanisms by which fermentation improves food safety and stability, has resulted in the development of new concepts for food preservation by using live microbial strains for bio-preservation. Moreover, health benefits of fermented foods have long been recognised, but the underlying mechanisms and the role of beneficial (functional) microbial strains have only been investigated and explored during the 20th century, leading to the development of the probiotic concept. Thus, the application of microorganisms in food processes relates to fermentation, either directly or indirectly. Appropriate strains, by virtue of their functionality, will exert beneficial effects, either on the food substrate and/or on the human host.

The intrinsic value of microbial strains for diverse applications in the food ecosystem and as genetic resources is appreciated and recognised in our time. Yet, mankind is still far from fully exploiting and utilising these valuable microbial resources. This study has been undertaken by the Handong Global University at the request of the Secretariat of the FAO Commission on Genetic Resources for Food and Agriculture and in close collaboration with the FAO Rural Infrastructure and Agro-Industries Division, to assess the status and trends of the conservation and sustainable use of microorganisms in food processes. A fully comprehensive study may have filled volumes and taken years, but the approach used was to:

- Compile and summarise key information on microbial strains relevant to food processing, also by referring to unpublished documents and reports, and putting together:
  - ✓ a detailed overview (in a comprehensive Table) of the types of microorganisms and their specific uses in food processes, and
  - ✓ information on the microbiology and other features of traditional fermented foods, worldwide, in several Tables, each giving information on a particular food raw material;
- Highlight major issues related to the sustainable use and availability of microbial strains for food processing,
- Present practical examples of successful applications (e.g., by case studies),
- Identify the potential for:
  - ✓ Exploiting existing resources, and
  - ✓ Networking and intensified concerted actions.
- Identify constraints related to communication and exchange, and also to point out bureaucratic and other hurdles.
- Recommend major actions that may be taken towards securing and supporting the sustainable use of microorganisms in food processes.

The “formal” food processing sector in industrialised countries is well organised. Large-scale enterprises generally have sufficient resources at their disposal, both for supporting R&D, and for securing sustainable use of modern technologies, including the controlled application of microbial strains in food processing. Moreover they can rely on the access to established culture collections (either internally or publicly) in which precisely characterised and defined microbial strains are being maintained. By contrast, the “informal” food processing sector is diverse and has been driven by basic needs, availability of raw materials, gradual development of technologies, and cultural traditions. Although not as well organised and sophisticated as the formal sector, these small-scale artisanal enterprises are producing a wide range of traditional fermented foods by which the basic needs of millions of people for safe and wholesome foods are being met. Also, there is an increasing appreciation of artisanal fermentation traditions providing a wide range of unique products (e.g.,

goat's and sheep milk cheeses) by which the culinary diversity, even in the market of industrialised countries, is enriched. Moreover, traditional food fermentations represent an extremely valuable cultural heritage in most regions, and harbour a huge genetic potential of valuable but hitherto undiscovered strains. Standardisation of traditional (artisanal) food fermentations may be hampered by various factors. Approaches to overcome basic constraints should include technical training and education of small-scale processors, and making available appropriate and affordable starter cultures.

The continuing and partly controversial debate on global warming and climate change has alerted authorities and politicians alike. The availability of safe, sufficient and wholesome foods may become an extremely critical and sensitive issue should these scenarios become a reality. Numerous indicators confirm regional climate changes on all continents, and particularly the increasing desertification is threatening the existence and traditional lifestyle of around 2 billion people, many of whom rely on traditional, small-scale food fermentations. Developing mathematical models may be of great value for predicting effects of changes in environmental conditions on microbial populations in traditional food fermentations.

I. INTRODUCTION

I.1. Background and rationale

This study has been prepared by the Handong Global University at the request of the Secretariat of the FAO Commission on Genetic Resources for Food and Agriculture (the Commission) and in close collaboration with the FAO Rural Infrastructure and Agro-Industries Division. The Commission, at its Twelfth Regular Session in July 2011, emphasized the need for assessing the status and trends of microorganisms relevant to food and agriculture. In this context, it requested, *inter alia*, the preparation of an analytical study on the status and trends of microorganisms in food processing. [3]

I.1.1. Global food and beverage sector

The global food and beverage (F&B) sector, comprising of farming, food production, distribution, retail and catering (Figure 1), was valued at 5.7 trillion USD in 2008 and is expected to increase at a Compound Annual Growth Rate (CAGR) of 3.5% to 7 trillion USD by 2014 (www.imap.com/imap/media/resources/IMAP_Food__Beverage_Report_WEB_AD6498A02CAF4.pdf).

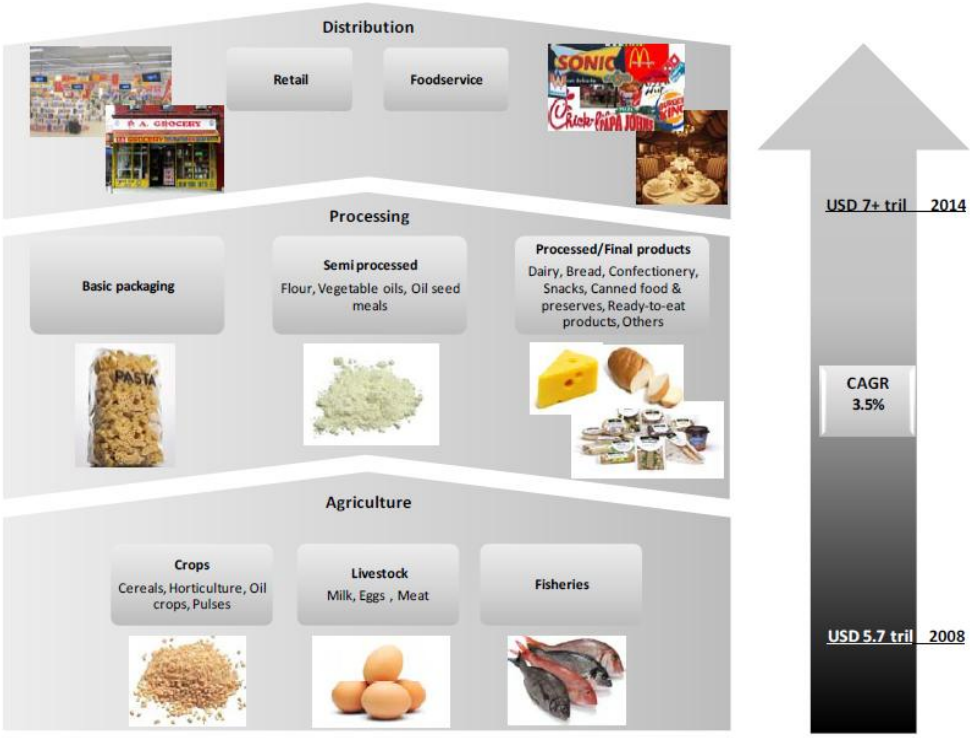

Figure 1. Food and beverage industry structure
Source:
www.blominvestbank.com/Library/Files/Uploaded%20Files/Final%20FB%20Industry%20Report.pdf

The economic slump has had an adverse impact on most industries including the F&B industry. The major problems faced by the industry are rising food prices, increasing transportation costs due to the rise in oil prices, and decline in consumer spending. Nevertheless, the F&B industry has been relatively less affected when compared to other industries. This is mainly attributed to the fact that food products continue to be essential to consumers in spite of the slowdown

[3] CGRFA-12/09/Report, paragraph 60.

(www.imap.com/imap/media/resources/
IMAP_Food__Beverage_Report_WEB_AD6498A02CAF4.pdf).

Basic agriculture is losing its value share in the global food products industry to processed foods. Out of the 3.2 trillion USD in revenues as of 2008, agriculture contributed 35% and packaged foods and meats accounted for the remaining 65%. Such a high share of packaged products is on account of a shift in consumer demand towards more value- added and processed products, brought about primarily by rising per capita incomes in both developing and developed countries (Figure 2).

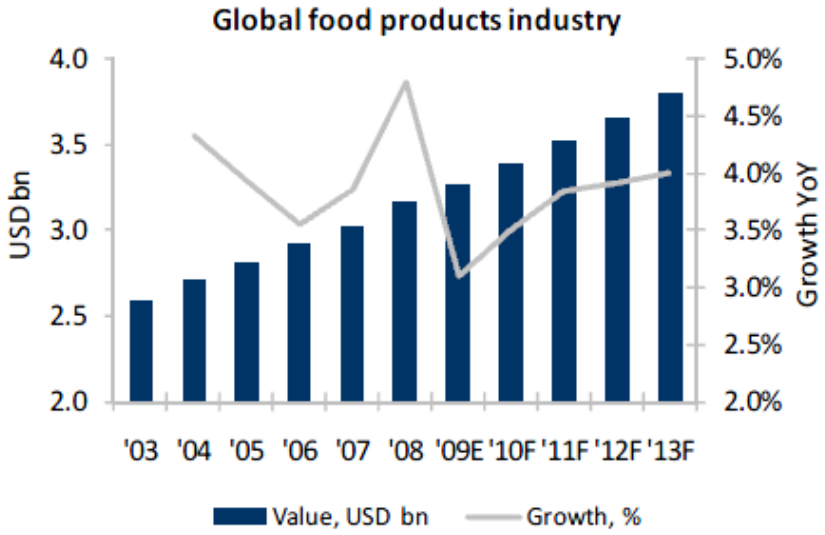

Figure 2. Value and growth (in %) of global food products industry from 2003 to 2013 (forecast)
Source:
www.blominvestbank.com/Library/Files/Uploaded%20Files/Final%20FB%20Industry%20Report.pdf

Among the various processed food groups, chilled and deli foods' revenues were the highest in 2008 at 385 billion USD, followed by dairy with 280 billion USD (www.blominvestbank.com/Library/Files/Uploaded%20Files/Final%20FB%20Industry%20Report.pdf). The fastest growth by far was recorded by the organic food segment, which grew at a CAGR of 14.6% between 2003 and 2008. Savoury snacks and dairy were the other two categories, which recorded a CAGR of above 6%. The global beverage industry, which consists of soft drinks, beers, ciders, spirits and wines, was valued at 1.4 trillion USD in 2008 and is expected to rise at a CAGR of 2.6% to 1.6 trillion USD by 2013. Within beverages, distillers and vintners accounted for the largest sales of 507 billion USD in 2008, followed by brewers at 494 billion USD. With sales of 387 billion USD, soft drinks represented the most valuable non- alcoholic beverage category. Bottled water was the fastest growing segment with a CAGR of 6.7% between 2003 and 2008, driven by the rising consciousness among consumers for clean and hygienic drinking water in a convenient form, in addition to being an alternative for other not- so- healthy drinks. Increasing health consciousness and convenience has contributed to the growth in the juices segment as well, which stood at a CAGR of 3.3% during the same period. In volume terms, the overall beverage market consumption grew at a CAGR of 6.9% between 2004 and 2008 to 9.9 billion liters (Figure 3) (www.blominvestbank.com/Library/Files/Uploaded%20Files/Final%20FB%20Industry%20Report.pdf).

Europe accounts for the largest share in the global F&B industry, generating revenues of 1.4 trillion USD in 2007, followed by the US, which contributed 1 trillion USD. However, Asia, led by China and India, is now emerging as a major contributor of raw material to the F&B industry. India's F&B market was valued at 182 billion in 2007-2008, while the food processing sector alone was worth 72 billion USD in 2008. China's food processing sector increased 13.6%, from 44 billion in 2007 to 50 billion USD in 2008.

Historically, developed countries such as the US, have been the largest producers of food products. However, there has been a slight shift in recent times, with China, Russia and India

increasing their production capacities. For example, in the case of wheat production, China increased its production capacity by 26% from 2003 to 2007, while Russia raised its capacity by 45%. In comparison, over the same period, wheat production in the US decreased by 12.5%.

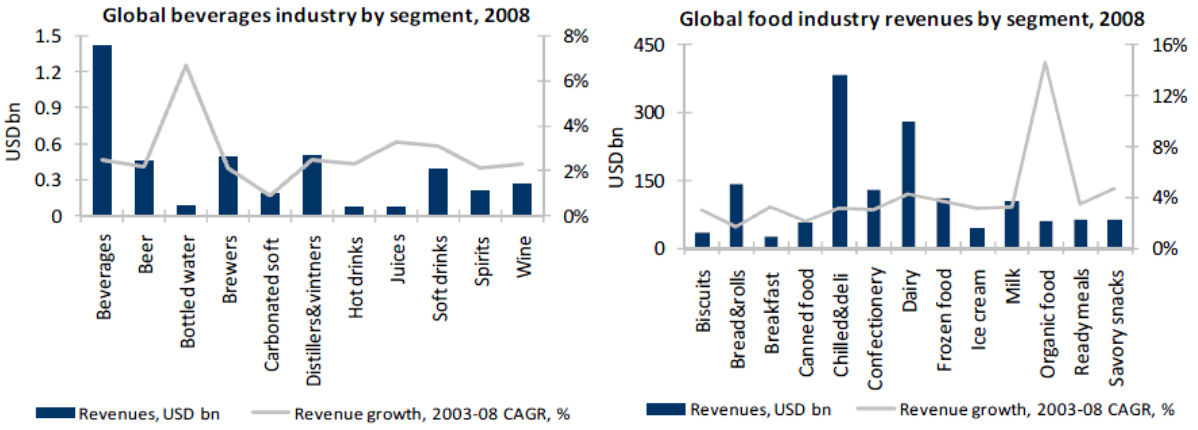

Figure 3. Global food and beverage industry revenues by segment, 2008
Source:
www.blominvestbank.com/Library/Files/Uploaded%20Files/Final%20FB%20Industry%20Report.pdf

Additionally, food purchases in developing countries are shifting from staple foods rich in carbohydrates to more expensive foods, such as meat and dairy products, indicating the significant growth potential of developing countries vis-à-vis developed economies. This trend is visible even on the consumption front. At present, about 58% of food produced is consumed by developing countries. It should be stressed that the majority of the increase in global population from 6.6 billion in 2008 to 9 billion in 2050 is expected to come from developing countries. The above percentage is expected to climb from 58% to 72% by 2050, supported by the fact that 37% of the world's population currently lives in China and India.

Demand for cereals is expected to rise from the current 2.1 billion tonnes to more than 3 billion tonnes by 2050, while demand for meat production is likely to grow by 200 million tonnes, to 470 million tonnes over the same period. Packaged food forms the majority of total food consumed, with developed countries, such as the US, Japan and EU, accounting for more than half of global sales of packaged products. However, raw products, which need to be processed before becoming edible, account for a large proportion of retail sales in developing countries. Nevertheless, with rising income levels, consumption in developing countries is also shifting towards packaged food products (www.imap.com/imap/media/resources/IMAP_Food__Beverage_Report_WEB_AD6498A02CAF4.pdf).

I.1.2. Overview of the use of microorganisms in food processing

Microorganisms constitute by far the largest group of living organisms on earth, with only a small fraction of microbial species having been identified up to now. They can be highly diverse in their biochemistry, physiology and nutritional modes. Most of them reproduce rapidly and the significant plasticity of their genome allows them to easily adapt to changing environmental conditions, as well as perform a variety of essential ecosystem functions, on which food production depends. According to FAO (2009a), the main functional groups for food processing are beneficial microorganisms (fermentation and probiotics). Microbial food cultures include bacterial food cultures, fungi and yeasts. These microorganisms determine the characteristics of the fermented food, e.g., acidity, flavour and texture, as well as health benefits that go beyond simple nutrition (Vogel *et al.*, 2011). These microorganisms may be present as natural indigenous microbiota of the food or as a result of the intentional addition of the microorganisms as starter cultures in an industrial food fermentation process (Stevens and Nabors, 2009). Also, microbial cultures can be used to produce

several compounds (enzymes, flavors, fragrances etc.) either specifically for application as food additives or *in situ* as a part of food fermentation processes (Longo and Sanromán, 2006).

I.1.2.1. Fermentation

Fermentation was traditionally a process, which served the preservation of perishable food and as such has been used for centuries until present (www.eolss.net/sample-chapters/c06/e6-34-09-09.pdf ; Hansen, 2004). As new preservation techniques have been developed, the importance of fermentation processes for food preservation has declined (Guizani and Mothershaw, 2007) and nowadays the main purpose of food fermentation is not just to preserve but also to produce a wide variety of fermentation products with specific taste, flavor, aroma and texture. Using various microbial strains, fermentation conditions (microorganisms, substrates, temperature, time of fermentation etc.) and chemical engineering achievements, it is now possible to manufacture hundreds of types of dairy (cheeses, fermented milk products), vegetable (sauerkraut, pickles, olives), meat products (fermented sausages) and bread, alcoholic beverages (wine, beer, cider), vinegar and other food acids, as well as oils. Historically, fermentation products were food products, but in recent years an increased interest has been observed towards the production of food additives (e.g. flavor modifiers) (www.eolss.net/sample-chapters/c06/e6-34-09-09.pdf).

Yet, fermentation is a relatively efficient, low energy, inexpensive preservation process, which increases the shelf life and decreases the need for refrigeration or other forms of food preservation technology. Therefore, it remains a very appropriate method for use in developing countries and rural communities with limited facilities. In addition, the non-dependence of fermentation on the use of chemical additives to the food appeals to the “more aware” consumer market (FAO, 1998; Guizani and Mothershaw, 2007). Fermented foods are popular throughout the world and in some regions make a significant contribution to the diet of millions of individuals. In Asia the preparation of fermented foods is a widespread tradition. The fermented food products supply protein, minerals and other nutrients that add variety and nutritional fortification to otherwise starchy, bland diets. For instance, Soy sauce is consumed throughout the world and is a fundamental ingredient in diets from Indonesia to Japan. "*Gundruk*" which is a fermented and dried vegetable product is very important for ensuring food security for many Nepali communities especially in remote areas. It is served as a side dish with the main meal and is also used as an appetizer in the bland, starchy diet. The annual production of *gundruk* in Nepal is estimated at 2,000 tones. *Gundruk* is an important source of minerals particularly during the off-season when the diet consists primarily of starchy tubers and maize, which tend to be low in minerals. In Africa, fermented cassava products (like *gari* and *fufu*) are major components of the diet for more than 800 million people and in some areas these products constitute over 50% of the diet. Because of the tremendously important role that artisanal, traditional fermentation procedures play in food preservation and their potential to contribute to the growing food needs of the world, it is essential that the knowledge of their production is not lost. There is a danger that the introduction of 'western foods' with their glamorous image will displace these traditional foods (FAO, 1998).

The term “fermentation” comes from the latin word *fermentum* (to ferment). The historical definition describes fermentation as the process, in which chemical changes in an organic substrate occur as the result of action of microbial enzymes (www.eolss.net/sample-chapters/c06/e6-34-09-09.pdf). Campbell-Platt defined fermented foods as “those foods, which have been subjected to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modification to the food” (Bamforth, 2005). However, in the stricter sense, the term ”fermentation” describes a form of energy-yielding microbial metabolism, in which an organic substrate, usually a carbohydrate, is incompletely oxidized, and an organic carbohydrate acts as the electron acceptor (Adams, 1990). Paradoxically, the term industrial fermentation usually refers to either aerobic or anaerobic processes, whereas fermentation in biochemical context describes a strictly anaerobic process. Industrial fermentation, a term used in chemical engineering, describes the process operations that utilize a chemical change induced by a living organism or enzyme, in particular bacteria, yeast, molds or fungi which produce a specified product (www.eolss.net/sample-chapters/c06/e6-34-09-09.pdf).

Fermentation has been used since ancient times to conserve and alter foods. The first fermentation included the production of beer (Babylonia), soy sauce (Japan, China), fermented milk

beverages (Balkans and Central Asia). Fermented beverages appeared in 5000 B.C. in Babylon, 3150 B.C. in Ancient Egypt, 2000 B.C. in Mexico and 1500 B.C. in Sudan (www.eolss.net/sample-chapters/c06/e6-34-09-09.pdf). For thousands of years fermentation processes were carried out without understanding microbial mechanisms, as fermented foods were spontaneously fermented by autochthonous strains. Although it was invented long before the discovery of microorganisms, it seems that it arose more by accident than by guided efforts. The microbial and enzymatic processes responsible for the transformations were largely unknown. It was understood that some processes required an inoculum, and the need for this was satisfied by keeping a sample from the previous production, frequently called "back-slopping" (Holzapfel, 2002). This procedure is still in use for propagation of sourdough for private use, and also for the production of some artisanal cheeses. For other processes, inoculation was not necessary because naturally occurring microorganisms in the raw materials could, under proper conditions, be a reliable source of the microbiota. This is the case in the production of raw milk cheeses, wine, sauerkraut and some fermented sausages (www.eolss.net/sample-chapters/c06/e6-34-09-09.pdf; FAO, 1998; Hansen, 2004; Mogensen *et al.*, 2002b).

Although fermentation of foods has been in use for thousands of years, it is only recently that there has been a development in the understanding of these processes and their adaptation for commercialization. There is tremendous scope and potential for the use of microorganisms towards meeting the growing world demand for food, through efficient utilization of available natural food and feed stocks and the transformation of waste materials (FAO, 1998).

With the discovery of microorganisms, it became possible to understand and manage food fermentations. Methods for isolating and purifying microbial cultures became available in the 19th century. Sterilization or pasteurization of the raw materials prior to inoculation with well-defined cultures allowed the fermentation processes to be managed with little variation. The use of defined cultures became the industrial standard in breweries by the 19th century. During the 20th century, the wine, dairy and meat industries also shifted production procedures towards the use of well-characterised and defined starter cultures. In the beginning, starter cultures were isolates from earlier fermentations that were maintained and propagated at the site of production. Owing to problems in maintaining the quality of such undefined, multi-strain cultures, companies started to specialize, produce and maintain such cultures. However, attack, mutations and seasonal variations in composition of inoculation material (milk, vegetables, grapes, etc.) often made it difficult to secure stability of such multi-strain, wild type cultures. As a result, development of starters based on single strains or defined multi-strain starter cultures were initiated (Whitehead and Cox, 1935). The application of microbiology and process technology resulted in large improvements in the quality of the fermented food products. The quality improvements have been so great that today all significant production of fermented food is industrial, or at least professionally performed. The small amount of "home fermentations" conducted in the form of baking, home brewing and private cheese making usually rely on commercially available yeast and bacterial cultures. The maintenance of the microorganisms differs between the different food industries in the sense that some fermentation industries, such as breweries and vinegar producers maintain their own stains and inocula. In the dairy industry, as well as in the meat industry and bakeries, cultures are usually obtained from suppliers dedicated to the production of high quality food ingredients (Hansen, 2004; Mogensen *et al.*, 2002b).

Fermentation plays different roles in food processing (Bourdichon *et al.*, 2011). Major roles considered are:

1. Preservation of food through formation of inhibitory metabolites, such as organic acid (lactic acid, acetic acid, formic acid, propionic acid), ethanol, becteriocins, etc. often in combination with decrease of water activity (by drying or use of salt),
2. Improvement of food safety through inhibition of pathogens or removal of toxic compounds,
3. Improvement of the nutritional value,
4. Improvement of the sensory quality of the food, and
5. Expansion of the diet for more diversity.

Fermentations can basically be performed either by spontaneous fermentation, by back-slopping or by addition of starter cultures. By spontaneous fermentation the raw material and its initial treatment, will encourage the growth of an indigenous microbiota. For most spontaneous fermentations, a microbial succession takes place: quite often lactic acid bacteria (LAB) will initially dominate followed by various species of yeasts. Moulds will only grow aerobically, which limits their occurrence in certain types of fermented products. LAB produce lactic acid and other antimicrobial substances that will inhibit the growth of harmful bacteria along with reducing the sugar content, thereby prolonging the shelf life of the product. Yeasts mostly produce aroma components and alcohols. When molds are involved in fermentation, they generally contribute by producing both intra- and extracellular proteolytic and lipolytic enzymes that highly influence the flavor and texture of the product (Josephsen and Jespersen, 2004).

In back-slopping, a part of a precious batch of a fermented product is used to inoculate the new batch. This procedure produces a higher initial number of beneficial microorganisms than found in raw material and ensures a faster and more reliable fermentation than occurs in spontaneous fermentation. This procedure probably also favors the growth of bacteria producing antimicrobial substances, ensuring the growth of the same bacteria every time. Examples of back-slopping are home-made fermentation of milk, vegetables and cereals. Bread production made with sourdough is often also done by back-slopping (Josephsen and Jespersen, 2004).

Addition of starter cultures is most often used when it is possible to inactivate the indigenous microbiota by heat treatment of the raw material, permitting the growth of only the added starter microorganisms. However, it is not always possible to heat-treat the raw material (e.g. fruits and vegetables) without influencing the texture of the final product. Nevertheless, the addition of starter cultures – especially those containing a bacteriocin-producing strain alone or in combination with selected bacteriocin-resistant strains - may in fermentation of plants yield a greater possibility that the desirable microbiota will dominate in the fermentation. Single-strain starter cultures are primarily used for yeasts and moulds in the production of beer and wine, and LAB for the production of a few dairy products, sausages and sauerkraut. Multiple starter cultures are used for dairy products, sourdough, sausages and wine. Mixed undefined bacterial starter cultures, also called traditional or artisanal starters, are primarily used in the dairy industry and in sourdough production (Josephsen and Jespersen, 2004).

Novel insights into the metabolism of LAB offer perspectives for the application of a new generation of starter cultures. Functional starter cultures are starters that possess at least one inherent functional property. The latter can contribute to food safety and/or offer one or more organoleptic, technological, nutritional, or health advantages. The implementation of carefully selected strains as starter cultures or co-cultures in fermentation processes can help to achieve *in situ* expression of the desired property, maintaining a perfectly natural and healthy product. A functional LAB starter culture is able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, useful enzymes, or nutraceuticals, while-the so called probiotic strains, mainly LAB, exhibit health-promoting properties. Such functionalities also lead to a wider application area and higher flexibility of starter cultures (Leroy and De Vuyst, 2004). Nowadays, there are many commercial suppliers of starter cultures worldwide, such as Alce and CSL (Italy), ASCRS (Australia), Chr. Hansen and Danisco (Denmark), CSK and DSM (Netherlands), Degussa and Gewürzmüller (Germany), Lallemand (Canada), NZDRI (New Zealand), Quest International (UK), Rhodia (France) etc. All companies can easily be found on the World Wide Web (Hansen, 2002; 2004).

Apart of being used as starter cultures in food processing, microorganisms are significant source of several compounds that are used as food additives, such as enzymes, flavors, fragrances, bacteriocins etc. The largest group of industrial enzyme used in the food industry today is derived from microbial fermentation. All three classes of microorganisms (bacteria, fungi and yeasts) are sources of commercial enzymes. The process begins with revitalisation of the preserved culture and the gradual buildup of cell numbers through a multi-series inoculum development culminating in the seed fermenter. At this stage, the culture is ready for full fermentation where maximum growth of the microorganism and enzyme production takes place. The final stages of manufacture consist of downstream processing steps aimed at recovery of maximum amounts of the enzyme produced during fermentation (Piggott, 2002).

I.1.2.2. Food additives generated by microorganisms

An "Inventory of Processing Aids" has been prepared by the Codex Committee on Food Additives and Contaminants, and is being updated from time to time (CODEX, 1999; 2003; 2010). The objectives of the Committee are to develop information on substances used as processing aids and to identify processing aids whose safety should be evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Inventory of Processing Aids catalogues substances that are used in food solely as processing aids as defined by the Codex Alimentarius Commission; one specific inventory deals with enzymes (CODEX, 2010). EFSA (2009) has published an opinion on a dossier on Food Enzymes for Safety Evaluation by the "Scientific Panel of Food Contact Material, Enzymes, Flavourings and Processing Aids".

Microbial rennets from various microorganisms (marketed under the trade names such as Rennilase®, Fromase®, Marzyme®, Novoren®, Hannilase®, Suparen®, etc.), which are cheaper than animal rennet (chymosin), are being marketed since the 1970s and have proved satisfactory for the production of different kinds of cheese. Although the proteolytic specificities of the three commonly used fungal rennets are considerably different from those of calf chymosin, these rennets have been used to produce acceptable cheeses (Adler-Nissen, 1987; Neelakantan *et al.*, 1999; Claverie Martin and Vega-Hernandez, 2007).

Lactase preparations from *Aspergillus niger*, *Aspergillus oryzae*, and *Kluyveromyces lactis*, which are considered safe because these sources already have a history of safe use and have been subjected to numerous safety tests, present a wide range of applications. Low-lactose or lactose-free food is essential for lactose-intolerant people. Another advantage of lactase-treated milk is the increased sweetness of the resultant milk, thereby avoiding the requirement for addition of sugars in the manufacture of flavoured milk drinks. Manufacturers of ice cream, yoghurt and frozen desserts use lactase to improve scoop and creaminess, sweetness, and digestibility, and to reduce sandiness due to crystallization of lactose in concentrated preparations. Cheese manufactured from hydrolysed milk ripens more quickly than the cheese manufactured from normal milk (Neelakantan *et al.*, 1999).

Microbial lipases, which are region-specific and fatty acid specific, are of immense importance and could be exploited for retailoring of vegetable oils. Cheap oils could also be upgraded to synthesize nutritionally important structured triacyl-glycerols, like cocoa butter substitutes, low calories triacylglycerols and oleic acid enriched oils. Lipases have also been used for addition to food to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known flavour and fragrance compounds. Lipases have earlier been used in production of leaner meat, such as in fish. The fat is removed during the processing of the fish meat by adding lipases and this procedure is called biolipolysis. The lipases also play an important role in the fermentative steps of sausage manufacture and also to determine changes in long-chain fatty acid liberated during ripening. Earlier, lipases of different microbial origin have been used for refining rice flavour, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine (Hasan *et al.*, 2006; Treichel *et al*., 2010; Sangeetha *et al*., 2011).

Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. Current applications include the flavor enhancement of cheeses, the acceleration of cheese ripening, the manufacturing of cheese-like products, and the lipolysis of butterfat and cream. The free fatty acids generated by the action of lipases on milk fat endow many dairy products, particularly soft cheeses, with their specific flavour characteristics. Thus the addition of lipases that primarily release short chain (mainly C4 and C6) fatty acids lead to the development of a sharp, tangy flavour, while the release of medium chain (C12, C14) fatty acids tend to impart a soapy taste to the product. In addition, the free fatty acids take part in simple chemical reactions by being converted by the microbial population of the cheese. This initiates the synthesis of flavour ingredients, such as acetoacetate, beta-keto acids, methyl-ketones, flavour esters and lactones. A whole range of microbial lipase preparations has been developed for the cheese manufacturing industry: *Mucor meihei* (Piccnate, Gist-Brocades; Palatase M, Novo Nordisk), *Aspergillus niger* and *Aspergillus oryzae* (Palatase A, Novo Nordisk; Lipase AP, Amano; Flavour AGE, Chr. Hansen) and several others. Lipases also play a crucial role in the preparation of so-called enzyme modified cheeses (EMC). EMC is a cheese that is incubated in the presence of enzymes at elevated temperature in order to produce a concentrated flavour for use as an ingredient in other products (dips, sauces, dressings, soups, snacks, etc.) (Hasan *et al.*, 2006).

The use of enzymes enables bakeries to extend shelf-life of breads, enhance and control non-enzymatic browning, increase loaf volume and improve crumb structure. Bio-Cat Inc., Enzyme Industry, Troy, VA, offer product line of enzymes that can aid in these functions and more. Lipases from *Aspergillus niger*, *Rhizopus oryzae*, *Candida cylindracea* are used in bakery products. Millbo S.p.a. (Italy) manufactures a wide and continuously evolving range of bakery enzymes to fulfill the needs of the bakery trades. It supplies lipases (M 300LF), which are effective in replacing partially or totally the emulsifiers, and to increase the volume in bread and bakery (Hasan *et al.*, 2006).

Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks. The commercial protein hydrolysates are derived from casein (Miprodan; MD Foods, Viby, Germany), whey (Lacprodan; MD Foods) and soy protein (Proup;, Novo Nordisk, Bagsvaerd, Denmark) (Gupta *et al.*, 2002). The enzymes used in the food industry include Alcalase ®, Neutrase®, Esperase®, Protamex™, and Novozym ® FM. These enzymes are commercially marketed by Novozymes, Denmark. These bacterial proteases are used for improving the functional, nutritional and flavour properties of proteins. Neutrase® is a bacterial protease from *Bacillus amyloliquefaciens*, which is used in alcohol production for improving yeast growth. In baking, it is used to degrade proteins in flour for biscuits, crackers and cookies. In brewing, it is used for extracting sufficient proteins from malt and barley and for obtaining the desired level of nitrogen nutrients. It is also involved in lactose reduction and flavour modification in dairy applications. Acid protease from *Aspergillus saitoi*, aspergillopepsin I, is commercially marketed as Molsin F by Kikkoman Corp., Japan. The enzyme is useful for the production of seasoning materials from the foods containing various proteins, the degradation of the turbidity complex resulting from protein in fruit juices and alcoholic liquors, and the improvement of quality of protein-rich foods. Flavourzyme™ is a fungal complex of exopeptidases and endoproteases derived from *Aspergillus oryzae* used for extensive hydrolysis of proteins. Kojizyme™ is a similar complex, which finds application in the fermentation of soy sauce. These enzymes are also products of Novozymes, Denmark (Adler-Nissen, 1987; Sumantha *et al.*, 2006).

The largest industrial application of microbial ***pectinases*** is in fruit juice extraction and clarification. Pectins contribute to fruit juice viscosity and turbidity. A mixture of pectinases and amylases is used to clarify fruit juices. It decreases filtration time up to 50%. Treatment of fruit pulps with pectinases also showed an increase in fruit juice volume from banana, grapes and apples. Pectinase treatment accelerates tea fermentation and also destroys the foam forming property of instant tea powders by destroying pectins. They are also used in coffee fermentation to remove mucilaginous coat from coffee beans. Pectinolytic enzymes added to macerated fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristics (colour and turbidity) as compared to the untreated wines (Jayani *et al*. 2005).

Glucoamylase and ***β-amylase*** are used commercially in the production of low calorie beer. The basic idea is to use more dilute wort (the liquor which is fermented) but, by adding enzymes, to increase the range of carbohydrates in it, which can be fermented. The same level of alcohol as in normal beer may thus be reached in the fermentation with a lower concentration of residual carbohydrate. Huge quantities of microbial enzymes are consumed by the starch industry, first and foremost for the production of sweet, high fructose syrups from starch. The saccharification of dextrins to glucose is carried out by soluble glucoamylase from *Aspergillus niger* (Adler-Nissen, 1987).

Transglutaminase obtained from microbial fermentation has been applied in the treatment of food of different origins. Food treated with microbial transglutaminase appeared to have an improved flavour, appearance and texture. In addition, this enzyme can increase shelf-life and reduce allergenicity of certain foods (Zhu *et al.*, 1995).

The efficiency of ***xylanases*** in improving the quality of bread has been seen with an increase in specific bread volume. This is further enhanced when amylase is used in combination with xylanase. Xylanase are used concurrently with cellulase and pectinase for clarifying must and juices, and for liquefying fruits and vegetables. α-L-Arabinofuranosidase and β-D-glucopyranosidase have been employed in food processing for aromatizing musts, wines, and fruit juices (Beg *et al.*, 2001).

Flavours comprise over a quarter of the world market for food additives. Most of the flavouring compounds are produced via chemical synthesis or by extraction from natural materials. However,

recent market surveys have shown that consumers prefer foodstuff that can be labelled as natural. Plants have been major sources of essential oils and flavours but their use depends on natural factors difficult to control such as weather conditions and plant diseases. An alternative route for flavour synthesis is based on microbial biosynthesis or bioconversion. Several microorganisms, including bacteria and fungi, are currently known for their ability to synthesize different aroma compounds (Couto and Sanromán, 2006). In addition, microorganisms have historically played an integral role in the elaboration of the flavor components of many different foods through fermentation process. Products such as wine, vinegar, beer, fermented vegetables, milk, soya and meat have been preserved, modified and flavored by means of microbial strains (Longo and Sanromán, 2006).

Although many microbial processes have been described as being able to produce interesting flavours, the number of industrial applications is limited. A reason for this in most cases is the low yield. The microbial flavours are often present only in low concentrations in the fermentation broths, resulting in high costs for down-stream processing. This is compensated by the fact that the market price of natural aromas is 10-100 times higher than that of synthetic aromas. A number of flavour compounds are already produced by microorganisms on an industrial scale (Janssens *et al*., 1992).

BASF (Germany) produces 4-decalactone, a peach aroma, which is distributed by its subsidiary company Fritzsche, Dodge & Olcott. The process involves the bioconversion by *Yarrnwia lipoIytica* of castor oil, which is pressed from the seeds of *Ricinus communis* and is composed of 80% of ricinoleic acid. In the UK, (R)-delta-dodecanolide is prepared by Unilever on a commercial scale using baker's yeast and starting from 5-keto-dodecanoic acid. The lactone produced can be applied as a butter flavour in margarines. Butyric acid and ethyl butyrate are produced microbiologically by the American company Hercules Inc. *Clostridium butyricum* converts glucose under anaerobic conditions into butyric acid. Butyric acid, a component naturally present in butter and some cheeses, can be applied for instance as a natural cheese aroma. Esterification with ethanol gives rise to ethyl butyrate, an important fruity flavour. Several processes use *Penicillium roquefortii* for the production of methylketones, which are important flavours of blue-veined cheeses. Methyl-ketones are produced on a commercial scale by the British company Stafford Speciality Ingredients, using a strain of *Aspergillus niger*. In this process coconut oil is converted into a mixture of methylketones during fermentation on a solid culture medium (Janssens *et al*., 1992).

I.1.2.3. Protective cultures

Biological preservation refers to the extension of the shelf-life of food products and improvement of their microbial safety by using two different approaches:

- the inoculation of the food matrix with microorganisms, defined as protective cultures, with consequent *in situ* production of inhibitory molecules and/or a competitive effect against pathogen and spoilage bacteria and
- the use of microbial metabolites in purified form, in particular bacteriocins.

The application of pure bacteriocins in food has several drawbacks, the major of which is the reduced efficacy determined by the binding to food components (fat or protein particles) and food additives. On the contrary, the use of microorganisms as protective cultures, e.g. bacteriocin producers, may have several advantages, as microorganisms can not only be the source of anti-microbial peptides but also of a wide spectrum of molecules, such as organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl, whose antimicrobial action is well known. Competition of protective cultures with potential pathogens is another way to restrict the growth of undesired organisms. Moreover, these microorganisms may have additional functional properties and, in some circumstances, they can be beneficial for the consumers. Last but not least, they can contribute to the flavour, texture and nutritional value of the product. Therefore, the concept of "protective cultures" is a broad one and it is not strictly related to the production of bacteriocins (Gaggia *et al*., 2011).

The term "Protective Cultures" has been applied to microbial food cultures (MFC) exhibiting a metabolic activity contributing to inhibit or control the growth of undesired microorganisms in food. These undesired microorganisms could be pathogenic or toxigenic bacteria and fungi but spoilage causing species may also be included. These cultures are considered as an integral part of starter

cultures, which are the traditional tools of food technology used to produce fermented food. It is a general property of fermented foods that possess a longer shelf life than the non-fermented raw materials. This property is the result of the active metabolism of the fermenting culture, conducting its actions through a complex system of competition for nutrients and binding sites and by production of inhibitory metabolites. Those starter culture species apart of being used in fermentation processes have also been applied to food in order to make use of the "bioprotective" potential with or without sensory impact (Holzapfel *et al*., 1995). Their usage is not limited to "classic" fermented foods but also plays an important role when their metabolic activities take place in food with a neutral pH and high water activity, which are subject to increased risk of growth of food pathogens (EFFCA, 2011).

Protective cultures should in the first instance be considered as additional safety factor, with the potential of improving the microbiological safety of food. Their implementation should support good manufacturing practices, thereby reducing risks of growth and survival of pathogens and spoilage organisms. In addition, under abuse conditions of temperature, handling, etc., their metabolic activities (e.g. acid or gas production) may serve as an indicator of microbial risk (Holzapfel *et al*., 1995; 2003).

LAB represent the microbial group most commonly used as protective cultures, as they are present in all fermented foods and have a long history of safe use. Involved in numerous food fermentations known to man for millennia, it is assumed that most representatives of this group do not pose any health risk to man, and some are designated as "GRAS" ("generally recognized as safe") organisms. Besides safety, protective cultures should guarantee the absence of detrimental effects on the target food. Since LAB may contribute to spoilage in several types of foods, it is essential to study their effect on food texture and quality, with particular emphasis on the nutritional value of the product. Furthermore, the capability of surviving to industrial processing conditions is of great importance for industries producing protective cultures at the large scale. On the other hand, in a number of product groups, especially dairy products, the use of biological preservation may also contribute to the health benefits of a product, e.g. as for acidophilus milk. Health traits, such as stabilization of gastro-intestinal tract, anti-carcinogenic action and tumour control, may serve as important additional advantage for future selection and application of protective cultures (Gaggia *et al*., 2011; Holzapfel *et al*., 1995). LAB have historically been used as preserving agents in a number of fermented foods. However, the role of LAB as protective cultures has also been evidenced in several non-fermented foods (inoculation on the surface of the food matrix) including meat, plant and seafood products, aimed at the increase of microbial safety and quality, as summarized in Table 1 below.

Table 1. Recent applications of live protective cultures in non-fermented foods aimed at increasing food safety (Gaggia *et al*., 2011)

Food products	Target microorganisms (pathogens or spoilage)	Protective culture employed
Meat		
Chicken meat	*S. enteriditis, Li. Monocytogenes*	*Ent. faecium* PCD71
Beef meat	*Enterobacteriaceae*, *Pseudomonas* spp., *Bro. Thermosphacta*	*Lb. sakei* CETC 4808
	Spoilage LAB, *Bro. Thermosphacta*	*Lb. curvatus* CRL705
Ham	*Listeria* spp.	*Lb. sakei* 10A
Fruit and vegetables		
Iceberg lettuce	*S. enteriditis* Typhimurium, *Staph. aureus, Li. innocua.*	*Pseudomonas putida* LTH 5878
	S. enteriditis Typhimurium, *E. coli, Li. Monocytogenes*	*Leuc. mesenteroides* CM135, CM160, PM249

Golden delicious apples	*S. enteriditis* Typhimurium, *E. coli, Li. Monocytogenes*	*Leuc. mesenteroides* CM135, CM160, PM249
Non-fermented pickles	*Li. Monocytogenes*	*Lb. curvatus* LR55
Seafood		
Cold-smoked salmon	*Li. monocytogenes*	*Cb. divergens* V41,
	Li. Innocua	*Lb. casei* T3, *Lb. plantarum* Pe_2,
		Cb. piscicola Sal_3
	Vibrio spp., *Salmonella* spp.	*Leuc. gelidum* EU2247
Cooked and fresh peeled shrimp	*C. botulinum, Staph. aureus*; *Shewanella putrefaciens*, *Photobacterium phosphoreum*, *Aeromonas* spp. *Pseudomonas* spp.	*Lact. piscium* EU2441

Protective cultures find application in several foods. In fresh fermented products, soft cheeses, smear type cheeses such as Yogurt, Mascarpone, Anthotyro, Brie they are used to control spoilage microorganisms and Listeria and in semi-hard and hard cheeses such as Cheddar, Gouda, Kaseri, Pecorino to control spoilage microorganisms. In cured, cooked and ground meat products they are used to control *Listeria, Cambylobacter* and *Salmonella* (www.pathogencombat.com/workshop/~/media/Adtomic/PatComBilleder/Articles/Brussels/BrusselsBiavati.ashx). A strain combination of lactic acid bacteria (LAB) and propionic acid bacteria (PAB), used as protective culture, was found to be the most active against yeasts, molds and *Bacillus* spp. in fermented milks and in bakery products (Suomalainen and Mäyär-Mäkinen, 1999). A novel application of protective cultures is their use on seafood products. Unlike meat or dairy products, seafood products are mainly non-fermented. Pathogenic microorganisms (*Vibrio*, *Listeria monocytogenes* and histaminogen bacteria) and spoiling microbiota are not always reduced or limited by the processing steps that are currently used for these foodstuffs. The addition of bacterial cultures concept, even with protective effects, is new and probably not totally yet accepted by seafood producers, for which the main goal is to avoid bacterial contamination by the use of good hygienic practices. However, in lightly preserved fish products (LPFP), the use of protective culture is gradually considered as an alternative to the use of food additives and it is gaining interest in the seafood industry. Among the microbial population of lightly preserved seafood products, lactic acid bacteria usually become dominant during the storage under vacuum or modified atmosphere. In some cases these bacteria are responsible for spoilage but some of them have demonstrated potential for pathogenic or spoiling microbiota inhibition. Some bacteriocins of these bacteria have been tested mainly for the control of *Listeria monocytogenes* in cold smoked salmon and to a lesser extent in other products to enhance sensory shelf-life. Many successful results have been obtained at the laboratory scale; nevertheless, the application in seafood industry is still limited. However a protective culture is currently used in seafood. This starter named LLO is applied in France for extending the shelf-life of cooked shrimp stored under Modified Atmosphere Packaging (MAP) and has also showed limitation of histamine production in Tuna stored at 5°C (Pilet and Leroi, 2011).

Industrial starters like SafePro® (CHR Hansen, DK) and Bovamine Meat CultureTM (NPC, US) have been developed for *L. monocytogenes* control for the meat industry (Pilet and Leroi, 2011). HOLDBACTM (Danisco, DK) find several applications in efficient spoilage and pathogen prevention in fermented dairy and meat products. It is used for growth control of yeasts and moulds and some heterofermentative lactic bacteria in fresh fermented foods, for growth control of leuconostoc, heterofermentative lactobacilli and enterococci in hard and semi-hard cheese and for growth control of

Listeria in soft and smear cheese, dry and semi-dry cured meats, cooked and fresh ground meats (www.danisco.com/product-range/cultures/holdbactm/). Microgard™ (Wesman Foods, Inc. Beaverton, USA) is the pasteurized product of the fermentation of skim milk by *Propionibacterium freudenreichii* spp. *shermanii* and its protective action has been associated with diacetyl, propionic, acetic and lactic acid and probably due to a heat stable peptide. It inhibits Gram-negative bacteria such as *Pseudomonas, Salmonella* and *Yersinia* as well as yeasts and moulds. It has been approved by the FDA for use especially in Cottage cheese and fruit flavoured yoghurt. Another commercial product is Bioprofit™ (Valio, Helsinki, Finland) which contains *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* JJ. The product is reported to inhibit yeasts and moulds in dairy products and *Bacillus* spp. in sourdough bread. ALTA™ 2341 (Quest International, USA) is produced from *Pediococcus acidilactici* fermentation and has to rely on the inhibitory effects of natural metabolites, including organic acids and the bacteriocin pediocin. When used can serve as an effective barrier to help control the development of *Listeria* in dairy products. ALC 01 (Niebüll, Germany) is also a patented antilisteral culture developed especially for soft cheese production. Its protective activity is due to pediocin generated by *Lactobacillus plantarum*. It inhibits the growth of *Listeria* on the surface of artificially and/or naturally contaminated Munster cheese after spray treatment. FARGO™ 23 (Quest International, USA) contains the same metabolites as for ALTA™ 2341, but live culture producing pediocin is present in greater quantity. In France it is added to raw milk intended for raw milk cheese production (Kesenkas *et al.*, 2006).

Bacteriocins are defined as proteinaceous compounds (usually peptides) that have bactericidal action against a range of organisms, which are usually closely related to the producer organism (Barnby-Smith, 1992). For LAB this fact implies two main disadvantages: that bacteriocins produced by protective cultures may inhibit other desired starter cultures and that are not active against Gram-negative pathogens and spoilage bacteria. The site of bacteriocins action is the cytoplasmic membrane (Holzapfel *et al.*, 1995). Bacteriocins can be used in food processing as additives, as part of the hurdle technology. For instance, nisin is commercially made in a partially purified form and a marketed preparation with the pediocin PA-1 (AcH) producer is available (De Vuyst and Leroy, 2007). In fact, nisin, one of the earliest bacteriocins to be described, is the only one that is commercially exploited at present. This is due to its relatively wide spectrum of activity, the stability of the genes that encode its production, and the stability of the purified bacteriocin (Barnby-Smith, 1992). It is produced by strains of *Lactococcus lactis* subsp. *lactis* and inhibits Gram-positive and Gram-negative bacteria and also the outgrowth of spores of Bacilli and Clostridia (de Arauz *et al.*, 2009). More specifically, nisin is added to processed pasteurized cheese to prevent late blowing (gas bubble formation) by clostridia, as clostridial spores, often present in the raw milk, can survive pasteurization. Nisin may also be added to low-acid (higher than pH 4.5) canned foods, such as vegetables, to prevent the growth of heat-resistant spores of *Clostridium thermosaccharolyticum* and *Bacillus stearothermophilus*, both of which can survive the heat treatment designed to kill *Clostridium botulinum* spores. Other more recent applications of nisin include prevention of the spoilage of beer and wines by lactic acid bacteria. The limited spectrum of activity of nisin can be exploited to good effect in beer, since yeasts are insensitive to nisin, and thus nisin can be added during fermentation. Nisin can also be added to wines to control the malolactic fermentation by preventing growth of the natural lactic acid bacteria, or, when this fermentation is required, by adding a nisin-resistant strain of lactic acid bacteria (Barnby-Smith, 1992).

As an alternative to the addition of bacteriocins to foods, bacteriocins may be produced directly in the food as a result of starter culture or adjunct culture activity. Several studies have indeed indicated that LAB starter cultures or adjunct cultures are able to produce their bacteriocins in food matrices, and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacteria. The latter trait has mainly been documented for fermented sausage, fermented vegetables and olives, and dairy products (De Vuyst and Leroy, 2007). For example, pediococci are commercially important in the fermentation of meats and vegetables. The bacteriocins from *Pediococcus* spp. are of interest because they inhibit a wider range of pathogens than many other bacteriocins. The inhibitory spectrum includes *Listeria monocytogenes* and the Gram-negative organisms *Pseudomonas fragi* and *Pseudomonas fluorescens*. Pediocin PA-1, produced by *Ped. acidilactici* strain PAC 1.0, displays antimicrobial activity against a wide spectrum of Gram-positive bacteria, many of them responsible for food spoilage or food borne diseases, and shows a particularly strong activity against *Listeria*

monocytogenes (Barnby-Smith, 1992; Rodríguez *et al.*, 2002). Pediocin AcH, from *Ped. acidilactici*, has been shown to inhibit *L. monocytogenes*, *Staph. aureus* and *Clostridium perfringens*. Pediocin A, produced by *Ped. pentosaceus*, has potential as a food preservative, owing to its relatively wide spectrum of activity, which includes *Clostridium* spp. and *Staph. aureus* (Barnby-Smith, 1992). Another example is the bacteriocins produced by the genus *Enterococcus*, the enterocins. Many of these enterocins have been found to be active against *Listeria monocytogenes*, and a few have also been reported to be active even against Gram-negative bacteria, an unusual property for the bacteriocins produced by LAB (Khan *et al.*, 2010).

I.1.2.4. Probiotics and functional foods

In the industrialized world, the concepts in nutrition have changed significantly during the last 60 years. From an initial emphasis on survival, through hunger satisfaction, and a subsequent focus on food safety, food sciences now aim at developing foods to promote well-being and health by conferring beneficial physiological and psychological effects that go beyond adequate nutritional effects. Existing scientific data show that both nutritive and non-nutritive components in foods have the potential to modulate specific target functions in the body, which are relevant to well-being and health and/or reduction of some major chronic and degenerative diseases, such as cardiovascular diseases, obesity, gastrointestinal tract disorders and cancer.

Thus, the era of functional foods started emerging. The term "functional food" was first used in Japan, in the 1980s, for food products fortified with special constituents that possess advantageous physiological effects. Today, functional foods constitute worldwide the fastest growing sector in the food industry. They largely represent healthier versions of mainstream foods and drinks, and thus allow consumers to eat and drink more healthily without radically changing their diet. The principal food types in this market are functional breakfast cereals, cereal bars and breads, Columbus eggs, functional fruit juices, cholesterol-lowering margarines, and last but not least yoghurt and yoghurt drinks with probiotic bacteria.

The term probiotic has been defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002). The development of probiotics during the past decade has signalled an important advance in the food industry transferring to towards the development of such foods (Ouwehand *et al.*, 2002; Saad *et al.*, 2012). Recently, both in Japan and Europe the market of functional food is dominated by gut health products, in particular probiotics. The global market for probiotic ingredients, supplements, and foods is expected to reach $19.6 billion in 2013, with more than 500 probiotic products introduced in the past decade alone (Ghishan and Kiela, 2011; Siró *et al.*, 2008).

Probiotic organisms used in foods must be able of surviving passage through the gut; i.e., they must have the ability to resist gastric juices and exposure to bile. Furthermore, they must be able to proliferate and colonize the digestive tract. In addition, they must be safe and effective, and maintain their effectiveness and potency for the duration of the shelf-life of the product (FAO/WHO 2002; Saad *et al.*, 2012). Microbes from many different genera are being used as probiotics. The most commonly used strains belong to the heterogeneous group of lactic acid bacteria (*Lactobacillus, Enterococcus*) and to the genus *Bifidobacterium* (Ouwehand *et al.*, 2002; Saad *et al.*, 2012). However, other microbes and even yeasts have been developed as potential probiotics during recent years (Ouwehand *et al.*, 2002).

At present, probiotic bacteria are mainly incorporated into dairy products, such as cheese, yogurt, ice cream and other dairy desserts. Fermented dairy products are the most traditional source of probiotic strains of lactobacilli; however, probiotic lactobacilli have been added to cooked pork meat products, snacks, fruit juice, chocolate and chewing gum (Bernardeau *et al.*, 2006; Ouwehand *et al.*, 2002; Ranadheera *et al.*, 2010). Limitations of dairy products, such as the presence of allergens and the requirement for cold storage facilities, as well as an increasing demand for new foods and tastes have initiated a trend in non-dairy probiotic product development. Further, it is important to develop probiotic products with food and beverages that are part of day-to-day normal diet to maintain minimum therapeutic level easily (Ranadheera *et al.*, 2010). Beverages would be the next food category where the healthy bacteria will make their mark. Likely candidates are chilled fruit juices,

bottled water, or fermented vegetable juices (Prado *et al.*, 2008). Fruit juice has been suggested as a novel, appropriate medium for fortification with probiotic cultures because it is already positioned as a healthy food product, and it is consumed frequently and loyally by a large percentage of the consumer population (Siró *et al.*, 2008).

The beneficial effects of probiotic foods on human health and nutrition are increasingly recognised by health professionals. Scientific work on the properties and functionality of living microorganisms in food have suggested that probiotics play an important role in immunological, digestive and respiratory functions, and that they could have a significant effect on the alleviation of infectious diseases in children and other high-risk groups. For example, many investigators have evaluated the therapeutic effects of LAB commonly used in yoghurt production against diseases such as cancer, infection, and gastrointestinal disorders. In parallel, the number and type of probiotic foods and drinks that are available to consumers, and marketed as having health benefits, has increased considerably (FAO/WHO 2002; de LeBlanc *et al.*, 2007).

I.2. Scope of the study

Although it is widely known that beneficial microorganisms, such as those used in food fermentations as well as probiotics, are very important in food processing, the current status and trends in their uses and conservation are not well documented, especially for the countries in the developing economies. This is in spite of the importance of microorganisms in food processing being generally well appreciated.

For the purpose of this study, two major categories of application of microorganisms in foods may be distinguished, i.e.

- As processing aid, e.g., starter cultures, playing an essential role due to their metabolic activities, and thus constituting an integral part of the technical process, and
- As additives, e.g. probiotics (with little or no metabolic activity in the food).

Yet, such a categorisation may be arbitrary, as the distinction between a processing aid and an additive is not always clear. For example, a probiotic strain may also perform essential metabolic activities (fermentation) within the food substrate, and thus may be considered as “multifunctional”.

According to an EU Council decision, a food additive is defined in Community legislation as “any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods” (Council Directive 89/107/EEC; EU, 2004).

There is a need to study and collect information on the diversity of microorganisms in order to develop well-informed strategies to expand and enhance the utilization of microorganisms in food processing, where appropriate. Also, while the use of microorganisms in food processing, such as during fermentation is well established, the current uses, opportunities and challenges that microorganisms have in enhancing other food processing needs to be better understood and documented. Furthermore, there is a need to investigate the current status and trends in the use and conservation of microorganisms in traditional food processing. That is due to the fact that traditional food processing methods, such as fermentation, are important activities in traditional food processing where microorganisms play a major role. It is also essential to investigate the use of microorganisms in traditional food processing, especially in the developing world, where they play an important role in small-scale food industries, and in rural development as a whole.

In addition to the above, there is a need to explore the effects that climate change poses to the diversity of microorganisms which are used in food processing, to enable the development of appropriate strategies to mitigate those effects. This is necessary owing to the fact that while

microorganisms are highly beneficial in food processing they are also prone to the effects of climate change.

I.2.1. Objectives

The main aim of the study is to assess the current status and trends of the use and conservation of microorganisms in food processing, with a focus on the use of microorganisms in different forms such as cultures, enzymes, flavours, fragrances and additives. The study explored technologies associated with the use of microorganisms and at the same time investigated the technological needs required to further enhance the utilization of microorganisms in food processing.

The specific objectives of the present study were to:

- Identify and document the current status and trends of the use of microorganisms in food processing including those used in traditional food processing methods.
- Explore the relevancy of the need for diversity of the microorganisms.
- Identify the main microorganisms that are used in food processing and identify their specific uses in food processing.
- Explore and identify any conservation practices including traditional management practices that are used to safeguard this diversity.
- Explore any gaps in terms of knowledge, technologies and policies that could contribute to the improved use of microorganisms in food processing.
- Establish existence of any threats or opportunities to the use of microorganisms in food processing.
- Explore past, current and potential future impact of climate change on the use of microorganisms in food processing.

I.2.2. Methodology

The present study is essentially based on the collection of existing information. Initially, an extensive and in-depth review of the available information has been conducted. The review included available reports, project documents and other scientific literature and data relevant to the study. Information was gathered from both private and public sectors. Additional information and data were used from previous work experience in this domain derived from the Agricultural University of Athens, and the Federal Research Centre for Nutrition in Karlsruhe, Germany, and furthermore, information and data originated from other institutions or stakeholders. Few relevant examples of the on-going or recently completed activities in the areas of the enhancement of the use of microorganisms in food processing are also reported on. Questionnaires addressed to food producing companies, biotechnology companies and public services sector were used in order to obtain a complete view on uses of microorganisms in food processing.

II. THE CURRENT STATUS AND TRENDS IN THE USE OF MICROORGANISMS IN FOOD PROCESSING

II.1. Status of knowledge on the roles and uses of microorganisms in food processes, including traditional food processing methods

The uniqueness of several microorganisms and their often unpredictable nature and biosynthetic capabilities, given a specific set of environmental conditions, have rendered them candidates in attempts to solve difficult problems in life sciences and other fields (Gomes *et al.*, 2007). In the food industry, a great number of different microorganisms are used for the production not only of fermented

foods but also of several other microbial products used as food additives such as organics acids, amino acids, vitamins, enzymes, flavors etc. (Rani and Soni, 2007). Successful cultures based on modern technology have already found application in the areas of probiotics, bioprotection and general improvement of yield and performance for the existing culture market. More new cultures have been introduced for fermenting other food products (Hansen, 2002). For example, lactic acid bacteria (LAB) are widely used in the manufacture of fermented foods and are among the best studied microorganisms. These organisms are used in a variety of ways including food production, health improvement and production of macromolecules, enzymes and metabolites. Detailed knowledge of a number of physiological traits of the LAB has opened the way to novel applications of these organisms in the food industry, while other traits might be specifically beneficial for human health (Giraffa and Carminati, 2012; Gomes *et al.*, 2007).

Presently, due to a variety of tools that provide advanced molecular differentiation of microorganisms, microbial populations can be quantified and new microbial species isolated and identified (Giraffa and Carminati, 2012)., In addition, considering the vast microbial diversity, it is only safe to assume that many more microorganisms and their applications in food industry are waiting to be discovered and expoited.

II.1.1. Main types of microorganisms and their specific uses in food processing

The types of microorganisms used in the various food processes can be grouped as follows:

- **Bacteria:** genera *Acetobacter, Arthrobacter, Bacillus, Bifidobacterium, Brachybacterium, Brevibacterium, Carnobacterium, Corynebacterium, Enterobacter, Enterococcus, Gluconacetobacter, Hafnia, Halomonas, Klebsiella, Kocuria, Lactobacillus, Lactococcus, Leuconostoc, Macrococcus, Microbacterium, Micrococcus, Oenococcus, Pediococcus, Propionibacterium, Staphylococcus, Streptococcus, Streptomyces, Tetragenococcus, Weisella, Zymomonas.*

- **Fungi:** genera *Actinomucor, Aspergillus, Fusarium, Lecanicillium, Mucor, Neurospora, Penicillium, Rhizopus, Scopulariopsis, Sperendonema.*

- **Yeasts:** genera *Candida, Cyberlindnera, Cystofilobasidium, Debaryomyces, Dekkera, Hanseniaspora, Kazachstania, Galactomyces, Geotrichum, Guehomuces, Kluyveromyces, Lachancea, Metschnikowia, Pichia, Saccharomyces, Schizosaccharomyces, Schwanniomyces, Starmerella, Torulaspora, Trigonopsis, Wickerhamomyces, Yarrowia, Zygosaccharomyces, Zygotorulaspora.*

Table 2 shows some examples of microorganisms that are widely used for the production of fermented and functional foods, microbial additives etc.

Table 2. Examples of microorganisms used in various food processes

Group / Genera	Major application(s)	Reference
Bacteria		
Acetobacter	Vinegar, Cocoa, Coffee, Vegetables	Bourdichon *et al.*, 2011
Bifidobacterium	Fermented milks, Probiotic properties	Mogensen *et al.*, 2002b

		Bourdichon *et al.*, 2011
Carnobacterium	Meat	Bourdichon *et al.*, 2011
Gluconacetobacter	Cocoa, Coffee, Vinegar	Bourdichon *et al.*, 2011
Lactobacillus	Dairy, Meat, Fish, Vegetables, Probiotics, Wine, Beverages, Sourdough	Mogensen *et al.*, 2002b Bernardeau *et al.*, 2006 Bourdichon *et al.*, 2011
Leuconostoc	Meat, Dairy, Vegetables, Wine, Fish, Coffee	Mogensen *et al.*, 2002b Hansen, 2004 Bourdichon *et al.*, 2011
Propionibacterium	Cheese fermentation	Mogensen *et al.*, 2002b Bourdichon *et al.*, 2011
Staphylococcus	Dairy, Meat, Fish, Soy	Mogensen *et al.*, 2002b Bourdichon *et al.*, 2011
Streptococcus	Dairy, Soy, Vegetables	Bourdichon *et al.*, 2011
Fungi		
Aspergillus	Soy, Beverages	Bourdichon *et al.*, 2011
Fusarium	Dairy	Bourdichon *et al.*, 2011
Penicillium	Dairy, Meat	Mogensen *et al.*, 2002b Bourdichon *et al.*, 2011
Rhizopus	Soy	Bourdichon *et al.*, 2011
Yeasts		
Candida	Dairy, Vegetables, Wine	Bourdichon *et al.*, 2011
Pichia	Wine, Dairy	Bourdichon *et al.*, 2011
Saccharomyces	Vegetables, Kefir, Beer, Bread	Mogensen *et al.*, 2002b Bourdichon *et al.*, 2011

A more detailed overview about the types of microorganisms and their specific uses in food processes is given in Table I of the Annex 1. Additionally, in Tables II to X (Annex 1), information on the microbiology and other features of traditional fermented foods, worldwide, is summarised. Each table gives information on a particular food raw material with regard to:

- Legumes – Table II,
- Cereals – Table III,
- Vegetables – Table IV,
- Sugary raw materials for Vinegar – Table V,
- Starch and juices for alcoholic fermentations – Table VI,
- Fish and seafood – Table VII,
- Fruit and root crops – Table VIII,
- Milk – Table IX,
- Meat – Table X

II.1.2. Use of microorganisms in food processing for the enhancement of the overall performance at small, medium and large-scale levels

The majority of small-scale fermentations in developing countries and even some industrial processes technically well controlled, such as sauerkraut fermentations and dill cucumbers, are still conducted as spontaneous processes (Holzapfel, 2002). Spontaneous fermentation represents an important food processing technology of low-cost, able to address the problems of food spoilage and food borne diseases, which are prevalent in the developing countries (Oyewole, 1997). In these fermentations indigenous microorganisms preserve food products, adding to their nutritive value, the flavour and other qualities associated with edibility. These processes are characterized by their limited need for energy input, allowing microbial fermentations to proceed without external heat sources (Achi, 2005).

Spontaneous fermentations typically result from the competitive activities of a variety of contaminating microorganisms. Those best adapted to the food substrate and to technical control parameters, eventually dominate the process. Bacteria typically dominate the early stages of fermentation processes, owing to their relatively high growth rate, followed by yeasts, in substrates that are rich in fermentable sugars. In numerous traditional processes, material from a previous successful batch is added to facilitate the initiation of a new process. Through this practice of back-slopping, the initial phase of the fermentation process is shortened and the risk of fermentation failure reduced. The indigenous microbiota in these fermentations contributes to a more complex sensory quality, whilst producing favourable synergistic effects, such as flavour production and accelerated ripening and maturation. However, there are various factors, which affect adversely the overall quality of traditional fermented foods, such as heterogeneity, since there is no consistence in the production due to the differentiation of the indigenous microbiota, unattractive appearance and lack of safety and increased hygienic and toxicological risks (Achi, 2005; Holzapfel, 2002)

It appears that as traditional fermented products become more popular and as demand grows, the only way, in which the expanding market can be satisfied, is to upscale the manufacturing process where the use of starter cultures becomes almost essential. This often has the consequence of diminishing the

uniqueness of the original product and the loss of those very characteristics that originally made the product popular (Caplice and Fitzgerald, 1999).

The characterization of the microorganisms responsible for the fermentation led to the isolation of starter cultures, which could be produced on a large scale to supply factories involved in the manufacture of these products. This significant development had a major impact on the processes used and contributed to ensuring consistency of product and reliability of fermentation (Caplice and Fitzgerald, 1999). Large-scale production generally now exploits the use of defined strain starter systems to ensure consistency and quality in the final product. Modern, large-scale production of fermented foods and beverages is dependent almost entirely on the use of defined strain starters, which have replaced the undefined strain mixtures traditionally used for the manufacture of these products. This switch to defined strains has meant that both culture performance and product quality and consistency have been dramatically improved, while it has also meant that a smaller number of strains are intensively used and relied upon by the food and beverage industries. This intensive use of specific starters has, however, some drawbacks and can lead to production problems resulting in unsatisfactory strain performance, e.g. bacteriophage infection (Paul Ross *et al.*, 2002).

II.2. Microbial diversity in food processing

II.2.1. Background

It is considered helpful to define a few terms relevant to biodiversity or "biological diversity". The Convention on Biological Diversity (CBD) defines "biological diversity" as "the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems". "Biological resources" includes genetic resources, organisms or parts thereof, populations, or any other biotic component of ecosystems with actual or potential use or value for humanity. "Biotechnology" means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify (http://www.cbd.int/convention/articles/?a=cbd-02).

Biodiversity reflects the "health" or vitality of an ecosystem and may be influenced by several factors including climate. This is exemplified by macroscopic plant and animal life in terrestrial habitats with tropical regions showing a richer degree of abundance and variation than the polar regions.

Species richness or species diversity are not considered to fully explain the complexity of the issue, and thus, biodiversity should reflect genetic diversity (totality of genes), species diversity (interspecies diversity or totality of species), and ecosystem diversity of a region (Hawksworth, 1996; Larsson, 2001, amended). Another level is the diversity (or variability) within a species (or intra-species diversity) (see below). The species concept is heavily debated among biologists. The critical issues deal with the parameters (or "minimal requirements") necessary for defining a new species, and when to call a newly isolated strain an "Operational Taxonomic Unit" (OTU). As a key parameter, the 70 % DNA-DNA hybridisation level of the total genome is considered a basic requirement in the species concept. The enormous metabolic flexibility, short generation time, occasional exchange of genes even across phylogenetic barriers (Torsvik and Øvreås, 2011) explains the unique ability of bacteria to adapt to diverse environments and ecosystems. Thereby, to some extent, the complexity and specific quality and other physico-chemical attributes derived from fermenting a food raw material into the final product may be explained.

Intra-species diversity (diversity within a species): Every species may contain numerous (even hundreds of) strains, each of which, when characterised, may differ in minor phenotypic (physiological) features (e.g. sugar fermentation pattern) from other strains of that species, but, as Operational Taxonomic Unit (OTU) still fulfils the minimal requirements for the particular species,

and in particular show > 70% genomic similarity to the type strain of the species. The basis for physiological variability among strains ("natural population") isolated from an ecological niche, such as a fermented food product, is their genetic variability (see "Molecular diversity" below). However, full information on intra-species genetic diversity may not be obvious. Functional diversity would usually be determined selectively according to requirements or expectations relative to the substrate and processing conditions, and may be based, e.g. on background information on functional genes and the molecular level techniques to monitor specific functional genes. Substrate specific gene expression at the transcriptional level may be decisive for assessing the functionality of a strain and its suitability (*i.e.*, superiority compared to other strains of the same species) for technical application.

Inter-species diversity: Several species of a genus may occupy an ecological niche, such as a fermented food product. Their intrinsic properties enable a species to successfully compete and adapt to a specific ecosystem or substrate for some time, but may be succeeded or outcompeted by another species that is better adapted to the modified conditions (change in pH, depletion of some nutrients, availability of newly synthesised growth factors, degradation of macromolecules, etc.).

Inter-genus diversity: A single genus will rarely occupy an ecosystem, e.g. during the traditional fermentation of food substrate. Several genera within one group (e.g. the LAB) of one prokaryotic domain, the *Bacteria*, may all contribute to the fermentation during different stages of the process. An example is the initiation of lactic fermentation in many vegetable fermentations by *Leuconostoc* spp. (e.g. *Leuc. mesenteroides*), followed by the domination (or increased activity) of *Lactobacillus* spp. (e.g. *Lb. plantarum, Lb. brevis, Lb. sakei*) and *Pediococcus* spp. (*Ped. pentosaceus, Ped. acidilactici*). In some vegetable fermentations, such as in kimchi, also *Weissella* spp. (*Weissella koreensis, Weissella cibaria*) have been detected in some samples. Many lactic fermentations may also be accompanied by contaminating organisms, such as yeasts and filamentous fungi, eventually causing spoilage. This represents a further level of microbial diversity within one niche, comprising the two domains, the *Bacteria* (prokaryotes) and the *Eukarya*, which includes the fungi and yeasts.

"Molecular diversity" can be considered as a highly specific dimension of biodiversity (Campbell, 2003, amended). This represents a basis for strain diversity within a microbial species where minor genetic diversity may be detectable. For the purpose of this study, strain diversity (within a species) is of paramount importance with an impact both on the typical features of product, and on the potential for industrial application. The Convention on Biological Diversity http://www.cbd.int/convention/articles/?a=cbd-01) has the objective to pursue "*the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources, including by appropriate access to genetic resources and by appropriate transfer of relevant technologies*." Operating under this Convention, the Global Taxonomy Initiative (http://www.cbd.int/gti/) [68], and also the European Distributed Institute of Taxonomy (http://www.e-taxonomy.eu), estimates the total number of species for some phyla to be much higher than estimated in 2010, now suggesting that there may be as many as 5 to 10 million bacterial species. This, amongst others, is based on the biological "richness" of seawater and on recent reports that one litre of seawater can contain > 20,000 different types of bacteria (http://news.bbc.co.uk/2/hi/science/nature/5232928.stm). The Prokaryotes comprise two of the three primary domains of life, the *Bacteria* and *Archaea*. The third domain, the *Eukarya*, include all living creatures having the eukaryotic cell type, i.e., plants, animals, protists and fungi.

The fungi, as an eukaryotic group of microorganisms, have been suggested in 2010 to include around 60,000 species (http://www.emc.maricopa.edu/faculty/farabee/biobk/ biobookdiversity_4.html; *retrieved 25.09.2012*), but even earlier, Hawksworth (2001) considered 1.5 million fungi as a conservative estimate of the real number. Data are still accumulating suggesting far higher numbers.

II.2.2. Definitions related to the food context

Various kinds of beneficial microorganisms are involved or implemented in food processing. Their diverse functions are summarised in Table 2. Definitions of these groups have been suggested by the

European Food and Feed Cultures Association (EFFCA). These definitions are appropriate and helpful for distinguishing the different applications of microbial cultures in food processing and preparation:

- Microbial Food Cultures (MFC) are preparations or formulations " *consisting of concentrates of one or more microbial species and/or strains including unavoidable media components carried over from the fermentation and components, which are necessary for their survival, storage, standardisation and to facilitate their application in the food production process*".

- Starter cultures are *"MFC preparations used as food ingredients in one or more stages in the food manufacturing process, which develop the desired metabolic activity during the fermentation or ripening process. They contribute to one or multiple unique properties of food stuff especially in regard to taste, flavour, colour, texture, safety, preservation, nutritional value, wholesomeness and/or health benefits*".

- Probiotic cultures are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host..". This definition is also in agreement with the joint FAO/WHO definition (WHO/FAO, 2001).

- Protective cultures are considered by EFFCA as "*an integral part of starter cultures rather than additives*", in line with the proposal by DG SANCO (2006). The capability of these cultures to develop their protective and beneficial potential is viewed as a result of their metabolic activity in or on the food, and thus resembles that of starter cultures. EFFCA views the principle inhibitory effect not to be the result of preformed metabolites in the culture preparation.

- Fermented foods relate to the term "fermented", describing "*the processes of acidification, maturing, ripening, flavouring, and preserving*". Therefore, the metabolic activity of the microorganisms in the preparations represents a "*fermentative event*".

- Labelling of food containing MFC describes MFC as a characteristic food ingredient, thereby requiring that products with MFC are to be labelled to show that they contain a "*microbial culture*".

II.2.3. The food ecosystem

From the outset of human civilisation, there has been an awareness of symptoms resulting from microbial interactions in foods as reflected through spoilage, food-borne diseases and ("spontaneous") fermentation.

Among the various methods applied for food processing (e.g., salting, drying, cooking), food fermentation has been one of the oldest and, traditionally, perhaps the best accepted by consumers. Studying traditional food fermentations {see also 2.1.} revealed that a wide range of beneficial microorganisms is involved in these fermentations. Rarely are these microbial populations dominated by a single strain; rather, they are involving a range of strains belonging to different microbial species and genera, and characterised by succession of various major population groups during fermentation. A fermentation may thus be characterised by the domination of one or more groups of microorganisms (diverse genera, species, strains), which are, in turn, influenced by factors such as food substrate, tradition (culture, experience, preferences), technological conditions, and climate (ambient temperatures, seasonal variations, etc.).

Fermentation thus represents the major background against which a wide variety of microorganisms find application, both in artisanal and industrial food production systems. These beneficial applications

are based on technical experience and, for industrial applications, also on information obtained from scientific studies on food fermentations. Beneficial microorganisms (see definitions above) are traditionally applied as starter cultures in food fermentations. They also find applications as probiotics in functional foods, for production of chemicals and enzymes for food production and as food processing aid, and for bio-preservation for preventing food spoilage and for combating health hazardous microorganisms.

Lactic acid bacteria (LAB) and yeasts are the two major microbial groups involved in food fermentations. In addition, *Bacillus* spp. and some moulds (fungi) may dominate fermentations of legumes and some other raw materials. Yet, one single species or strain will rarely solely dominate a fermentation process. This may be one reason why traditional starter cultures (see Table 3), applied for small-scale operations, are a valuable source of strain and species complexity, also for ensuring the typical characteristics of numerous traditional fermented foods.

Food fermentations may thus reflect a wide species and genus diversity of associated LAB (see Table I of Annex 1). Likewise, a diverse range of strains applied in industrial food processing has been derived either from food fermentations or the food environment, including the gastro-intestinal tract (GIT) for probiotic strains. While single-strain cultures are more clearly defined and predictable than mixed strain cultures, the latter may have advantages in a situation where either not all conditions are precisely defined, bacteriophage infection may be a threat to a strain (Émond and Moineau, 2007), or where more complexity in sensory properties and/or diverse interactions are required for obtaining the desired product.

Table 3. Some traditional starter culture products of the world

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	**Substrate**	**Sensory property and nature**	**Microorganisms**	**Country / references**
Budod	Rice, starter	Basi production	*Endomycopsis fibligera*, *Aspergillus* sp., *Rhodotorula* sp.,	Philippines
Binubudan (Binuburan, Purad)	Milled rice + Budod	Basi production	*Debarymyces hansenni, Cand. parapsilosis, Trichosporon fennicum*	Philippines
Binokhok	Roast rice	Starter	*No descrription?*	Philippines
Hamei	Rice	Starter	*Mucor* sp., *Rhizopus* sp., *Sacch. cerevisiae, Pichia anomala*	
Look-pang	Rice flour, powder of Kha root, spices	Starter, cake	*Rhizopus* sp., *Mucor* sp., *Chlamydomucor* sp., *Penicillum* sp., *Aspergillus* sp., *Asp. niger*	Thailand

			Asp. flavus, *Endomycopsis* sp., *Hansenula* sp., *Saccharomyces* sp.	
Marcha	Glutinous rice, roots, wild herbs, ginger, red dry chili	Starter	*Mu. circinelloides*, *Mu. hiemalis*, *Rhiz. chinensis*, *Rhiz. stolonifer*, *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis*, *Pichia anomala*, *Pichia burtonii*, *Sacch. cerevisiae*, *Sacch. bayanus*, *Cand. glabrata*, *Ped. pentosaceus*, *Lb. bifermentans*, *Lb. brevis*	India / Tamang *et al.*, 2012
Meju	Soybean	Fermented soybean starter	*Asp. oryzae*, *B. subtilis*	Korea
Murcha	Rice or wheat, wild plants	Starter	*Sacch. cerevisiae*, *Rhizopus* sp., *Endomycopsis fibligera*, *Ped. pentosaceus*, *Lb. plantarum*	Nepa, Bhutani
Nata de Coco, *Nata ng Niyog*, *Nata de Pina*	Coconut water, sugar starter	Nata	*Ac. xylinum*	Philippines
Nuruk (Kokja)	Wheat	Starter	*Asp. oryzae*, *Candida* sp., *Asp. niger*, *Rhizopus* sp., *Penicillum* sp., *Mucor* sp., *Hansenula anomala*, *Leuc. mesenteroides*, *B. subtilis*	Korea
Samac	Sugar cane	Basi production	Yeast, bacteria, moulds	Philippines
Ragi	Rice flour, spices	Starter	*Amylomyces* sp., *Mucor* sp., *Rhizopus* sp., *Endomycopsis* sp., *Saccharomyces* sp., *Candida* sp., *Pediococcus* sp., *Bacillus* sp.	Indonesia

II.2.4. Examples of microbial diversity

The study of traditional food fermentations has become an exciting "exploring field" of microbial diversity. With an estimated 5 000 varieties of fermented foods and beverages, worldwide, only a small fraction of these artisanal products have been subjected to scientific studies so far (Tamang and

Kailasapathy, 2010). The examples given below are therefore only an indication of an expected enormous microbial diversity, harbouring potentially valuable strains and resources of genetic diversity. The diversity within species can be exemplified for *Lactobacillus plantarum*, although other species may represent equally interesting examples of strain diversity.

II.2.4.1. Intra-species diversity

- ***Lactobacillus plantarum*** has one of the largest genomes (> 3,000,000 bp) among the lactobacilli. It is closely "related" to *Lactobacillus paraplantarum, Lactobacillus pentosus* (Torriani *et al.*, 2001) and *Lactobacillus fabifermentans* (De Bruyne *et al.*, 2009). The whole genomes of at least four *Lb. plantarum* strains have been sequenced (Axelsson *et al.*, 2012; Kleerebezem *et al.*, 2003; Siezen *et al.*, 2012; Wang *et al.*, 2011; Zhang *et al.*, 2009). The large genome may explain the „heterogeneity" of this species and its (strain specific) association with diverse ecological niches, including the plant phylosphere, the human gastrointestinal tract (GIT), vegetables, dough (cereals), meat and dairy fermentations. Its enormous diversity in phenotypic properties and metabolic capacity are strong indicators of its huge potential for industrial applications (Siezen and van Hylckama Vlieg, 2011). Selected strains, originally isolated from traditional fermentations, are used in industrial food fermentations in the meat, dairy and olive industry, as silage inoculants some of which are able to survive in the rumen (Weinberg *et al.*, 2003), and perform as a probiotic (e.g., the commercial strain 299V).

The intra-species diversity of *Lb. plantarum* strains from artisanal food fermentations has been reported in several publications, showing wide physiological (phenotypic) differences among strains. Although some strains can be accessed through institutional or governmental culture collections, these may represent only a minute fraction of the overall diversity.

Access to well characterised and technically (industrially) valuable strains may be an important requirement in support of innovative activities for small and medium enterprises. Large companies typically have their own R&D sections, maintaining their own (non-accessable) culture collections, and, in order to remain competitive and keep track with consumer expectations, applying sophisticated procedures to find new and interesting strains for biotechnical applications.

- **Maasai milk (*Kule Naoto*):** *Lactobacillus plantarum* was found to be the dominant species in Maasai traditional fermented milk *Kule Naoto*, and 76 of the 130 isolated *Lb. plantarum* strains were characterised and identified, some of which showed probiotic properties (Mathara *et al.*, 2008a). Further studies revealed that several strains showed a variety of functional and technical properties, suggesting the potential for industrial application and for improvement of quality and safety of existing traditional fermented food products in Kenya (Mathara *et al.*, 2004; 2008a; 2008b; Holzapfel, 2002). These strains were considered as good candidates for multifunctional starter cultures in addition to strains of *Lb. acidophilus, Lb. paracasei* and *Lact. lactis*, also isolated from Maasai fermented milk. The performance of these strains was compared in a model according to a Central Composite Design with regard to the effects of fat, non-fat milk solids, and fermentation temperature, and also considering fermentation rates, viability losses during refrigerated storage of the chosen starters, and product texture parameters (Patrignani *et al.*, 2006).

- **Two artisanal Italian cheeses** (*Fiore Sardo* and *Caciotta*): *Lactobacillus plantarum*-group (*Lb. plantarum, Lb. pentosus* and *Lb. paraplantarum*) strains isolated from the artisanal Italian cheeses, *Fiore Sardo* and *Caciotta*, were characterised on the basis of their phenotypic and genotypic profiles in order to obtain information on the ecology and biodiversity, and potential probiotic properties of „wild" (non-commercial) strains of this *Lactobacillus* group, and also with the view on potential industrial applications (Pisano *et al.*, 2011). Physiological and biochemical characteristics showed diverse phenotypic patterns among strains. In addition, while genotyping studies using randomly amplified

polymorphic DNA (RAPD)-PCR also revealed wide heterogeneity among the strains, but still enabled the distinctive grouping of the strains into the species *Lb. plantarum vs. Lb. pentosus/Lb. paraplantarum*. Four *Lb. plantarum strains* (2 isolated from each of *Caciotta* and *Fiore Sardo* cheeses, respectively), showed probiotic features and survived simulated gastrointestinal conditions. Only one strain was found to produce a bacteriocin-like compound.

- **Biodiversity of mannose-specific adhesion in *Lactobacillus plantarum*:** Some *Lb. plantarum* strains have been reported to show probiotic features, and the commercial strain 299v, claimed to have beneficial health properties, has found application in probiotic food products (De Vries *et al.*, 2006). Adhesion is considered to be an important property of probiotic strains; one mechanism of adhesion is by specific adhesin protein(s), providing an advantage for adaptation and colonisation in the GIT. A potentially 'probiotic' gene encoding the mannose-specific adhesin (Msa) of *Lb. plantarum* was identified by Pretzer *et al.* (2005), while the *msa* gene product has subsequently been shown to be the key-protein for mannose adhesion. When studying the structure and function of the *msa* gene and its genetic diversity in *Lb. plantarum* strains relative to the mannose adhesion capacity, Gross *et al.* (2010) found significant variation in the quantitative *in vitro* mannose adhesion capacity of these strains. This was supported by *msa* gene sequence variation. The strain-specificity of probiotic (and other) characteristics is illustrated by these results, and underlines the importance of exact (molecular and phenotypic) characterisation of candidate technical starters and probiotic strains.

- **Ecological distribution of enterococci, and diversity within the species *Enterococcus faecium***: As in the case of *Lb. plantarum*, the enterococci can be considered as "ubiquitous" organisms, and also with relatively large genome sizes for the LAB, varying from 2550 to 2995 kb for *Enterococcus faecium*, and with genome sizes of 3000-3250 kb reported for *Enterococcus faecalis*, 3445 kb for *Enterococcus avium*, and 3070 kb for *Enterococcus durans* (Oana *et al.*, 2002). The species *Ent. faecium* and *Ent. faecalis* are particularly widespread, and are:

* typically associated with the human gastro-intestinal tract (Devriese and Pot, 1995),
* also reported to have a high incidence in poultry and cattle,
* predominating in porcine faecal samples,
* varying in numbers in the faeces of pre-ruminant calves and ruminating young cattle,
* detected in the faeces of dogs and cats (Devriese *et al.*, 1992; Leclercq *et al.*, 1996),
* considered as a major cause of nosocomial infections, but are also
* commonly found in the food matrix and food environment of raw and processed meats,
* associated with fermented meat and dairy products (Ben Omar *et al*., 2004; Todorov *et al*., 2012; 2013),
* commercially used in fermented foods, e.g. in the fermented milk product Gaio®, distributed in Denmark and Sweden, for which functional (probiotic) properties are claimed (Bertolami and Farnworth, 2003), and
* commercially available (a few strains, especially of *Ent. faecium*) as probiotics, either as food or feed supplements, with a long history of safe use.

Strains of *Ent. faecium* are particularly considered important for desired ripening and product flavour development of artisanal cheeses in Greece, Italy and other Southern European countries (Tsakalidou *et al.*, 1993; Sarantinopoulous *et al.*, 2002). Moreover, studies on various traditional food fermentations have revealed that strains of *Ent. faecium* and *Ent. faecalis* are associated with practically all kinds of fermented foods (Holzapfel, unpublished data), and may even play an important role in numerous artisanal food fermentations, as has been shown for *Hussuwa*, a traditional fermented sorghum product from Sudan, in which enterococci constitute around 10 % of the total microbial population (Yousif *et al.*, 2005). Their association with traditional legume fermentations, dominated by *Bacillus subtilis*, has also been reported for these fermentations both in West Africa (Oguntoyinbo *et al.*, 2007) and in Asia (Tamang, 2010).

Concern has been expressed because strains of *Ent. faecium* and *Ent. faecalis* have been identified as causes of nosocomial infections; especially *Ent. faecalis* is considered to be a major cause of the increasing incidence of endocarditis, bacteraemia, urinary tract and neonatal infections (Franz *et al.*, 1999; 2001). Infections due to *Ent. faecalis* hitherto represented a factor 10 over those caused by all other *Enterococcus* species, although a shift related to the emergence of vancomycin-resistant enterococci may increase the ratio of *Ent. faecium* among clinical enterococcal isolates (Mundy *et al*., 2000; Vancanneyt *et al.*, 2002).

Although enterococcal strains isolated from food harboured some virulence traits, their incidence was generally higher in *Ent. faecalis* than *Ent. faecium* (Franz *et al.*, 2003; Franz and Holzapfel, 2004, 2006; Foulquié Moreno *et al.*, 2006). The ability of enterococci to take up and transfer antibiotic resistance genes, vertically as well as horizontally, caused concern about the association of entercoccal strains with the food environment and in particular with food fermentations (Vancanneyt *et al.*, 2002). In particular, the use of enterococcal strains as probiotics has been questioned (Franz *et al.*, 1999). Meanwhile, the safety and acceptability as probiotics of some strains of *Ent. faecium* with a long history of safe use, has been fairly well established and accepted. This is based on well established scientific data and long-term surveillance reports (Franz *et al.*, 2011).

Intra-species diversity was investigated by Oana *et al.* (2002) who constructed a physical map of the *Ent. faecium* ATCC19434 chromosome and compared the rRNA operons (rrn) with those of *Ent. faecalis* and other species. They reported that *Ent. avium* and *Ent. durans* contained six rrn operons as in *Ent. faecium*, as compared to four rrn operons for all *Ent. faecalis* strains.

Vancanneyt *et al.* (2002) studied the intra-species strain relationships of 78 susceptible and vancomycin-resistant *Ent. faecium* strains from humans, animals and foods, collected from different European countries. Using three genomic typing techniques, *viz*.: RAPD-PCR (four different primers; resulting in subclusters "R"), AFLP (two different primer combinations; grouped in subclusters "A") and pulsed-field gel electrophoresis (PFGE) analysis of *Sma*I patterns, they demonstrated strong intra-species diversity within *E. faecium* based on two genomic groups, each subdivided with either with four or three subclusters, respectively. The typing results were evaluated with regard to the origin of the strains, safety aspects, such as beta-haemolysis and glycopeptide antibiotic resistance, and bacteriocinogeny. Even when host specificity of *Ent. faecium* strains could not be confirmed, the subclusters of group I showed some correlation with origin, pathogenicity, and bacteriocinogeny of the strains. It is also of significance that all human clinical strains clustered in group I, and that all animal isolates and human clinical strains were allocated to a single subcluster of group I (R1 or A1), whereas nearly all the food strains grouped in three clusters (R2 to R4 or A2 to A4) of group I. The usefulness of these genomic approaches deserves more attention for the purpose of selection and application of beneficial strains for use in food processing.

Jurkoviè *et al.* (2007) studied the genetic diversity and the presence of plasmid DNA of 176 *Ent. faecium* strains isolated from Slovakian Bryndza cheese. PFGE of genomic macrorestriction fragments of the 82 strains (46.6 %) with plasmid DNA showed a relativelys high intra-species genetic diversity among the enterococci from Bryndza cheese. ERIC–PCR and plasmid profile analysis resulted in a

higher resolution than PFGE and (GTG)5–PCR, thereby showing an even higher intra-species diversity of *E. faecium* than suggested by PFGE.

Bhardwaj *et al.* (2011) studied the genetic diversity among 14 bacteriocinogenic strains of *Ent. faecium,* isolated from dairy foods and faecal samples, by RAPD-PCR, in addition to investigating the presence of virulence determinants, production of biogenic amines, susceptibility to various antibiotics, and evaluating for their *in vitro* probiotic potential. Tyramine as the only biogenic amine was produced by 9 strains, while no strain was resistant to all antibiotics. One strain (*E. faecium* FH 99) showed good BSH activity, highest ability to tolerate acid and bile, and was able to assimilate cholesterol from growth media.

Using high-quality draft genome data, Palmer *et al.* (2012) characterised two clades within *Ent. faecium* and identified characteristic traits such as variation in operons for cell wall carbohydrate and putative capsule biosynthesis. The extent of recombination between the two *Ent. faecium* clades was examined and two strains with mosaic genomes were identified. The species-specific traits showing a potential for advanced detection of medically relevant enterococci were identified. This may serve as a future basis for rapid identification of enterococcal species or lineages importance in clinical and environmental samples (Palmer *et al.*, 2012).

II.2.4.2. Inter-species diversity in traditional food fermentations

- **Sourdough**

A variety of sourdoughs are being used worldwide, mainly for bread making, but also for preparing different fermented cereal gruels and mixtures with other food substrates. Sourdoughs may therefore be the best example of age-old artisanal traditions that are being practised globally on all continents, both on household and industrial scale. At the same time, the microbiology and physico-chemical aspects of sourdough fermentations are being studied on an intensive scale by various expert research groups in different countries, thereby revealing deeper insights into the complex microbial diversity of this food ecosystem. Practically all sourdoughs are the result of artisanal technologies and experience, even when some sourdoughs have been commercialised for industrial use. Typically, a sourdough comprises a dynamic mixture of different LAB strains and yeasts; their ratios and relationships will be determined by substrate-related and extrinsic factors (e.g. temperature, atmosphere, humidity), and by the technical factors applied in the process of transfer, culture maintenance, application and storage. A wide range of cereals (see Table III in Annex 1 on cereal fermentations) may be used for sourdough fermentations, and are determined by the availability of the raw materials, tradition and regional preferences.

The approach suggested by Böcker *et al.* (1995) to classify sourdoughs in three different types, I, II, and III, has been of great value to categorise doughs with different properties, and to understand the complexity of a dynamic system used commonly on a daily basis. Information on the typical LAB and yeast populations of Types 0, I and II sourdoughs is given in Table 4.

Table 4. Categories of sourdoughs and associated LAB and yeast populations (Gänzle, 2006; slightly modified)

Type 0	Type I	Type II
Dominating species		

Lb. plantarum Yeasts: Bakery yeast *(Sacch. cerevisiae*	*Lb. sanfrancisensis* *Yeasts: Candida humilis* *(syn. Cand. milleri)*	*Lb. pontis,* *Lb. panis*
Frequently isolated		
Lb. brevis, Lb. curvatus, *Lb. sakei, Lact lactis,* *Ped. pentosaceus*	*Lb. alimentarius, Lb. brevis,* *Lb. fructivorans, Lb. paralimentarius,* *Lb. pentosus, Lb. plantarum,* *Lb. pontis, Lb. spicheri* *Leuc. mesenteroides, W. confusa* *Yeasts: Sacch. exiguus, Sacch. cerevisiae, Issatchenkia orientalis*	*Lb. acidophilus,* *Lb. crispatus,* *Lb. delbrueckii,* *Lb. fermentum,* *Lb. reuteri.*
Occasionally found		
Lb. acidifarinae, Lb. zymae	*Lb. hammesii, Lb. mindensis, Lb.nantensis, Lb. rossii,* *Pedicoccus spp., W. cibaria*	*Lb. amylovorus,* *Lb. frumenti, Lb. johnsonii,* *Lb. mucosae, Lb. paracasei,* *Lb. rhamnosus* Yeasts: *Sacch. cerevisiae, Issatchenkia orientalis*

"Type 0" sourdoughs are distinguished from types I, II and III by being prepared in a one-step, discontinuous process (3-24 h) with baker's yeast (Gänzle, 2006). They are used for producing bakery goods from wheat, and are essential for baking specialities such as *ciabatta* and *baguette*. However, even in commercial compressed yeast, the numbers of LAB may exceed 10^8 CFU/g , while, during extended incubation times of the dough, the LAB may multiply by up to 1000-fold, resulting in numbers 5 times higher than those of the yeasts.

Type I sourdoughs have been defined by Böcker *et al.* (1995) to distinguish continuously produced sourdoughs from those with a long incubation time (Type II), also because of differences in their microbial populations (see Table 13). Type I sourdough is used without the addition of yeast for bread making, but with different steps in dough preparation, and using different raw materials, e.g., for sourdough bread in Germany, San Franciso sourdough bread in the USA, and *pannetone* in Northern Italy.

Type II sourdoughs have been defined by Böcker *et al.* (1995) as continuously propagated liquid sourdoughs with longer incubation times than Type I doughs, and with elevated fermentation temperatures from 30 to 40 °C. Thereby, acid sensitive species such as *Lb. sanfrancisensis* are inhibited due to a pH value < 4.5, and species with a higher acid tolerance are selectively promoted.

Type III sourdoughs are defined as dried (dehydrated) sourdoughs in which *Lb. plantarum* and *Lb. brevis* are most frequently isolated while sensitive species, such as *Lb. sanfrancisensis* may be destroyed during the thermal dehydration process (Böcker *et al.*, 1995; Gänzle, 2006).

The following studies explain the complexity of sourdough fermentations, even under controlled laboratory conditions. They, however, also indicate chances and oppportunities for modifying process conditions and to modulate microbial activities, so as to achieve particular objectives, e.g. for quality improvement and to enhance health features of the product.

- Weckx *et al.* (2010) investigated the dynamics and succession of the LAB population in spontaneously fermented rye sourdoughs, and compared these with that of wheat and spelt sourdoughs using a multiphasic approach. Applying back-slopping daily, four spontaneous rye sourdough fermentations were conducted over 10 days. Using statistical analysis, the data for rye, spelt and wheat sourdoughs, partly obtained in a previous study, were compared. During the different steps of the fermentation, a clear but gradual shift in the heterogeneous population, differing for each batch (A to D) and comprising up to 10 and more species, was found. A stable microbial ecosystem developed in the rye sourdough ecosystems after 10 days, resulting in the domination of *Lb. plantarum* and *Lb. fermentum*. Ornithine and mannitol, shown to be positively correlated with rye sourdoughs, contributed to bacterial competitiveness right from the onset of sourdough production. On the other hand, a high degree of similarity was detected for wheat and spelt sourdoughs.

- Kitahara *et al.* (2005) investigated the composition of LAB and the diversity of *Lb. sanfranciscensis* strains in 5 different sourdoughs. From the 57 isolated strains 21, originating from all sourdoughs, were identified as *Lb. sanfranciscensis*, and the others as *Lb. plantarum, Lb. paralimentarius, Lb. fermentum, Lb. pontis, Lb. casei, W. confusa* and *Ped. pentosaceus*. Intra-species diversity of *Lb. sanfranciscensis* was investigated using ribotyping, resulting in 21 ribotyping patterns with four clusters, but with the type strain, *Lb. sanfranciscensis* JCM 5668^{T} independent of the others. There appeared to be a relation between the intra-species diversity of *Lb. sanfranciscensis* strains and the sourdough preparation.

- Gerez *et al.* (2012) evaluated the gliadin hydrolysis during wheat dough fermentation by a combination of *Lb. plantarum* CRL 775 and *Ped. pentosaceus* CRL 792, and compared the results with those effect by a cell free extract (CFE). (*Gliadins, along with, glutenin, play a role in the formation of gluten, and may be the cause of food allergies or food-derived „pathogenesis", resulting in severe immune response also known as coeliac disease*). Interestingly, in the study by Gerez *et al.* (2012) the CFE produced a greater (121%) increase in amino acid concentration than the combined *Lactobacilus* strains (70-80%), and appear to be related to LAB peptidases, active as exo-enzymes in the dough. This also underlines the health promoting effects of microbial strains typically associated with food fermentations such as sourdoughs.

- In another study focusing on positive health protective effects resulting from the deliberate use of functional strains for fermentation, Rühmkorf *et al.* (2012) investigated the influence of flour type and various other factors on the *in situ* production of different exopolysaccharides (EPS) in sourdough by three *Lactobacillus* strains (*Lb. animalis* TMW 1.971, *Lb. reuter*i TMW 1.106 and *Lb. curvatus* TMW 1.624). In view of the worldwide increase in gluten-related food allergies, such as coeliac disease, the development of natural baking aids for producing gluten-free bread is highly desirable. EPS are produced by several food grade bacteria, and can act as hydrocolloids to improve bread qualities, in particular for preparing gluten-free bread (Waldherr and Vogel, 2009; Galle *et al*., 2012). The studies by Rühmkorf *et al.* (2012) showed that high amounts of EPS could be achieved in gluten-free (buckwheat and quinoa) sourdoughs under optimised conditions, and by using carefully selected LAB strains.

- **Microbial diversity in fermented soybeans and other legumes**

As protein rich plant foods, legumes form an essential part of the global human diet. The alkaline fermentation is mainly based on the activity of *Bacillus subtilis* and other *Bacillus* spp., although in some regions (e.g. Indonesia), filamentous moulds are the dominating microorganisms. These fermentations constitute ancient practices in Asia and some regions in West Africa and yield products that are either being used as condiments or form a major part of the diet. The legume raw materials for fermentation depend either on their availability in a region, and/or on tradition and preferences. Soya beans serve as the major source (90 % of fermented legumes) but other raw materials include garden peas, different bean types, including black gram, green gram and French beans, black lentils, locust beans (and related indigenous raw materials typical of West Africa) (Tamang *et al.*, 2010). More details on legume fermentations are provided in Table II of Annex 1. Although *Bacillus subtilis* has been reported as the dominant bacterium involved in the fermentation, other *Bacillus* spp. such as *B. amyloliquefaciens, B. cereus*, and *B. licheniformis* have also been detected, e.g. in okpehe produced in Nigeria from *Prosopis africana* (legume) seeds, and thus suggesting the inter-species diversity in these kinds of products (Oguntoyinbo *et al.*, 2010). Moreover, by the traditional alkaline fermentation of the non-legume seeds of *Hibiscus sabdariffa* in West Africa, another *Bacillus* dominated condiment, known as bigalka in Burkina Faso, is produced and widely used. A study by Ouoba *et al.* (2008) reflects the interspecies diversity characterising this fermentation, comprising *Bacillus subtilis* as predominant organism followed by *B. licheniformis*, while strains of *B. cereus, B. pumilus, B. badius, Brevibacillus bortelensis, B. sphaericus* and *B. fusiformis* were also detected. Extensive studies have been conducted towards the development of *Bacillus* starter cultures for legume fermentations in several Asian and African countries (Kimura and Itoh, 2007; Ouoba *et al.*, 2003a). *B. subtilis* is used for *natto* production in Japan, and also fermentation of soya beans to *kinema* in India, Nepal and Bhutan and *thua nao* in Thailand are typically associated with *Bacillus* spp. (Tamang, 2010), and for *chungkookjang* of Korea (Park *et al.*, 2005).These products are all characterised by typical stickiness (Tamang 2010).

A number of studies have been undertaken to develop starter cultures for fermentation of indigenous legumes, such as the seeds of *Prosopis africana* (Oguntoyinbo *et al.*, 2007), and of the African locust bean (*Parkia biglobosa*) (Ouoba *et al.*, 2003b; 2007a,b).

The fermentation of African locust bean (*Parkia biglobosa*) for production of *soumbala* typical of West Africa (also known under different names, including *dawadawa* or *okpehe*), has been intensively studied in Burkina Faso. With the purpose of developing starter cultures, strains of *B. subtilis* and *B. pumilus* have been subjected to studies on their proteolytic and lipolytic activities (Ouoba *et al.*, 2003a,b), degradation of polysaccharides (Ouoba *et al.*, 2007a), production of volatile compounds in *soumbala* (Ouoba *et al.*, 2005), and antimicrobial properties (Ouoba *et al.*, 2007b). Strains of *B. subtilis* and *B. pumilus* selected as potential starter cultures were genotyped by PCR amplification of the16S-23S rDNA intergenic transcribed spacer (ITS-PCR), restriction fragment length polymorphism of the ITS-PCR (ITS-PCR RFLP), pulsed field gel electrophoresis (PFGE) and sequencing of the 968–1401 region of the 16S rDNA, revealing clear intra-species diversity.

Doenjang, a typical soybean paste of Korea, has a long history of use as a fortified protein source in the Korean diet. The traditional, mainly artisanal fermentation has hitherto been considered to be dominated by *B. subtilis.* Using next-generation sequencing, Nam *et al.* (2012) have analysed the microbial "community" of traditional Korean soybean pastes, and derived 17,675 bacterial sequences from nine local and two commercial brands of *doenjang* samples. In contrast to results formerly obtained by plating or conventional molecular biological methods, a **diversity of bacterial species** (in total 208!!) was detected, with each *doenjang* sample reflecting a region-specific bacterial community. The *Bacillus* species were in high abundance (58.3–91.6%) only in samples from the central region of Korea, while LAB (39.8–77.7 %) dominated the bacterial population of other *doenjang* samples. Microbial communities of commercial brands, on the other hand, contained simple microbial communities dominated by *Tetragenococcus* spp. and *Staphylococcus* spp., thereby showing resemblance to Japanese *miso*. It appears that starter culture inoculation may contribute to quality control and standardization of the product; however, it may be assumed that the "complexity" in terms of sensory and quality attributes associated with the traditional product may be reduced.

II.2.5. Conclusions

The practical examples given on microbial diversity represent only a few of numerous examples. The rapidly accumulating in-depth information on microbial communities, their structure, interactions, succession during the fermentation process, influence on the quality and safety of the resulting product, reveal the incredible complexity of practically all artisanal fermentations. Moreover, the use of up-to-date analytical methods such as next-generation sequencing enables high resolution in the discrimination of the roles of single strains and species in a fermentation process. Advantages of such detailed, "fine tuned" information may be most valuable for:

- selection of appropriate starter cultures or combinations of strains,
- understanding population dynamics (Alegria *et al*., 2011), synergism and the role of key controlling factors of quality (sensory, physical structure, texture, nutritional value, health/functional attributes , etc.) and safety,
- understanding specific stress responses and underlying mechanisms, as they may (e.g.) be related to the activation of a functional gene under particular processing conditions (intrinsic, extrinsic, implicit), and
- application in consumer-friendly, "natural" processing approaches such as bio-preservation as alternative to chemical or thermal preservation (see also Section 2.3).

II.3. Challenges and opportunities in the sustainable use of microorganisms in food processing

Worldwide, fermented foods play an important role in the life, diet and culture of practically all societies and communties. However, the numerous benefits of food fermentations are of special importance to traditional communities in rural areas, rendering these fermented food products an essential part of their diet, lifestyle, and security, in contrast to industrialised societies. The positive attributes of these foods are in fact a guarantee for the sustaining of health and wellbeing of numerous rural communities, and include (e.g.):

- biological enrichment (by improved bio-availability) of nutrients,
- improvement of sensory attributes,
- expansion of the diet,
- bio-preservative effects and improved food safety,
- degradation of toxic components (e.g., linamarin in bitter cassava), residues or contaminants (including mycotoxins),
- degradation of antinutritive factors (e.g., enzyme inhibitors, phytic acid),
- functionality of microbial strains, including probiotic features, and
- medicinal and therapeutic values (Holzapfel, 2002; Tamang, 2007).

These ethnic fermented foods[4] provide some guarantee for food security to people of these regions throughout times of famine, extreme environmental and climate conditions, and man-made disasters.

[4] The expression "ethnic fermented foods" is defined by Tamang (2010) as foods produced by ethnic people from locally available raw materials of plants using their native knowledge.

Furthermore they serve as a source of revenue for small-scale, artisanal processors, including those living in remote areas, for sustaining their livelihood. Ethnic fermented foods of the Himalayas, and many regions in Africa, Latin America and Asia, can be exploited for enhancement of regional economy and also for sustainable development and protecting the rich microbial diversity and genetic resources (Tamang, 2009; 2010). Some of the Himalayan and other kinds of ethnic fermented foods are traditionally appreciated for beneficial health effects and as traditional form of medicine (Mathara *et al.*, 2004), and also for improvement of taste and to impart aroma (Tamang, 2010).

The sustainable use of microorganisms in food processing is based on an interrelationship among several factors, with regard to:

- traditional technical knowledge,
- modern expertise and information,
- basic understanding of the microbial background of a fermentation and of Good Hygienic Practices (GHP),
- some experience with handling of microbial strains or cultures, even under crude conditions such as "back-slopping",
- application practices ("high" or "low" tech), and
- conservation of microbial strains (see also III), or, alternatively, at least the technical handling for keeping and storage of crude traditional starters.

On the other hand, regulatory measures may put restrictions on the commercial use of "novel" strains with a potential for artisanal applications:

- Before approval of a strain for food biotechnical use, authorities in industrialised countries generally require special documentation and a submission procedure for proving the safety and applicability of a strain.
- As a result of "globalisation", approval procedures, including requirements for proof of "functionality" and safety of novel microbial strains for technical application, may also be adopted in developing countries. An example of severe restrictions on industrial progress and developments in Europe is the rejection by EFSA (European Food Safety Agency, Parma, Italy) of 74 probiotic health claims, even after resubmission of dossiers (discussed below).
- Concern for public health is justified, but preventing or restricting practices and the use of microbial strains with a history of safe use, may severely limit progress in small- and medium-scale manufacturing processes. This may even place constraints on large enterprises, which, however, have means to overcome these hurdles, thanks to larger resources and their own R&D departments.

Far from presenting an exhaustive list, the following are examples of problematic issues related to the use of microorganisms in food processing, in particular referring to small-scale enterprises operating in food fermentation. These problems should be processed as challenges towards finding practical solutions:

- Worldwide decline in traditional practices or in using indigenous knowledge of food fermentation, harbouring a diversity of native microorganisms with unknown (or insufficiently studied) properties.

- Shift in agricultural practices.

- Urbanisation and popularization of fast foods.

- Food safety concerns of some ethnic foods.

- Climate change affecting agricultural production, and, by reduced post-harvest availability, putting constraints on traditional processing of ethnic foods.

- Developments of monoculture (single-strain) inoculation and ignoring the potential of mixed cultures and their contribution to typical attributes in traditional fermentation.

- Marginalisation of local producers of fermented foods and beverages by Government Agencies and financial institutions.

- Lack of coordination and networking among processors and providers relying on microbial systems for food processing.

II.3.1. Enabling and impeding factors in the use of microorganisms in food processing

The roles, uses and availability of defined (typed and taxonomically identified) strains of microorganisms have been discussed under 2.1. Thanks to the application of molecular techniques and advances in microbial taxonomy, the knowledge basis on precisely identified microorganisms in traditional fermented foods is rapidly increasing. Examples of interspecies and intraspecies diversity are given under 2.2. Information on various categories of fermented foods, summarised in Tables II to X (Annex 1), reflect the enormous amount of available knowledge. Still, these Tables only cover a small proportion of the total number of fermented foods worldwide, and also include numerous products of which the microbiota are only vaguely named (e.g., LAB and/or yeasts). However, more precise taxonomic information on species and strain diversity, microbial community structure and succession in traditional fermentation processes is accumulating in recent years, part of which is reflected in these tables.

Factors in favour of the sustainable use of microbial strains in food processing include the:

- global availability of characterised and defined strains in culture collections (see also 3.2, and 3.4), although this might be a limiting factor for small-scale processors due to economics of purchase and technical handling of pure strain cultures,

- strong and increasing knowledge basis on the microbiology of traditional food fermentations,

- knowledge and technical skills obtained by training, knowledge sharing and education, including increasing opportunities for networking, and

- support (by NGO's, EU, national developmental organisations) in the standardisation and application of traditional skills and methods in conventional, small-scale operations in rural areas needs.

Authentic strains of microorganisms and supporting services are provided by culture collections, as important resources for research and development. These well-documented strains are publicly available, although, generally, at some cost. National and most other culture collections of the public

sector are affiliated as members of the World Federation for Culture Collections (WFCC). WFCC is a multidisciplinary Commission of the International Union of Biological Sciences (IUBS) and a Federation within the International Union of Microbiological Societies (IUMS), and deals with the collection, authentication, maintenance and distribution of cultures of microorganisms and cultured cells. The WFCC keeps records of around 476 culture collections from 62 countries (http://www.wfcc.info/about/, retrieved 29.09.2012), of which more than 80 % belong to public sector entities such as universities and governments. It is of significance that all culture collections with major holdings in food and agriculture are of the public sector or semi-governmental (FAO, 2009b). More detailed information is given under Section 2.1.

Governmental and public research institutional culture collections may contain potentially valuable strains of a much wider diversity than those in the large culture collections. Giraffa (2009) reported on the screening of *Lb. plantarum* in food biotechnology, and compared the amount of strains available in culture collections and in research collections, estimating around 30 strains to be the largest number available in specialised culture collections, such as the BCCM-LMG collection in Belgium (with 36 strains), the Institut Pasteur in France (29) and the Moroccan Coordinated Collection of Microorganisms (36). The German DSMZ collection only mentions 15 strains, 2 of which are designated to the „related" *Lb. pentosus*, and one to *Lb. paraplantarum*. On the other hand, the collections of the Agriculture Research Council (CRA) in Italy hold around 300 identified strains of *Lactobacillus plantarum* in the CRA-FLC and 50 strains from dairy, olive and wine fermentations in the CRA-cluster collection. A further 300-400 strains are deposited in other Italian research institutions and are to be further characterised (FAO, 2009b).

Collaborative research[5] between Karlsruhe, Germany and Kenya on the microbiology of Maasai fermented milk (*kule naoto*) revealed a rich biodiversity and multi-functionality of LAB strains associated with this traditional product, constituting a major part of the Maasai diet. Around 130 strains from this research project have been deposited at the Max Rubner (formerly BFE) research collection. Among several publications, one paper reported on the diversity of *Lb. plantarum* strains associated with *kule naoto* (Mathara *et al*., 2008a). Joint publications (Mathara *et al*., 2004; 2008a, b; Patrignani *et al.*, 2006; 2007) have reported the major research results. This information may serve as basis for further research and development of standardised starter cultures for the Kenyan and East African dairy industry.

II.3.1.1. Enabling factors – Opportunities referring to available knowledge and ways for its further exploitation

Information given below reflects aspects of recent progress in scientific development and awareness with regard to traditional food fermentations in the Asian and African continent, both of which may be representative of numerous more examples worldwide.

- ***Asia: Himalayan traditional fermented foods***

A case study on the potential of traditional Himalayan fermented foods is presented in the Annex 2.

Development and commercialisation of Himalayan traditional fermented foods should consider the following important parameters:

- **Microbial genetic resources**

[5] A joint visit to the Maasai region of Kenya for the collection of samples from traditional Maasai milk formed the basis for research conducted in Karlsruhe, Germany, by a doctorate student (J. Mathara) from Kenya. This formed part of collaboration between WH (author; project leader, Germany), the University of Nairobi, and the Jomo Kenyatta University, Kenya. This research has widened, thanks to collaboration with the University of Bologna, Italy, where technical and modelling studies have been conducted with the aim of developing starter cultures; a few strains have also been deposited in the culture collection at Bologna University.

Microbes may constitute important genetic resources for diverse functional applications in food production, medicine, agriculture and environment management. In the traditional human environment, edible and culturally acceptable foods have been developed by using available natural resources. Traditional fermented foods and beverages represent important sources of microbial diversity in the Himalayas; several of these products have been extensively studied (see Tables II, IV and VI in Annex 1), and the functional microorganisms associated with ethnic foods were isolated, characterised and identified (see Annex for references). The diversity of microbial communities associated with these traditional fermented foods and beverages is reflected by the numerous genera and species identified, comprising various species of the LAB genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Tetragenococcus* and *Weissella.* In addition, representative strains of several *Bacillus* species, various yeasts and filamentous fungi have been detected.

Some strains possess protective and functional properties, rendering them potential candidates for use as starter culture(s) for production of traditional fermented foods with improved quality. In addition, some strains may have potential for production of enzymes, bioactive compounds and for other industrial uses. The establishment of a gene bank in the Himalayas is suggested for preservation and maintenance of these strains, and to enable further investigations.

- **Interpretation of "Ethno-Microbiology" in the Himalayas**

The majority of the Himalayan fermented foods can be considered essential for food and nutritional security of the region (Tamang *et al*., 2010). Traditional knowledge and experience are contributed by the Himalayan people towards the production and management of the available food bio-resources, thereby supplementing the food ecosystem and enhancing the regional economy. This model represents a basis for the commercialisation of some traditional Himalayan food commodities. One particular aspect constitutes the skills for producing and applying traditional starter cultures for (e.g.) alcohol production technique and using traditional distillation apparatus. The wide microbial diversity, ranges from filamentous fungi to enzyme- and alcohol-producing yeasts, and also functional Gram-positive and a few Gram-negative bacteria. In order to protect and further exploit this huge potential of genetic resources, introduction of ethnic fermented foods in the syllabus at the master level in Microbiology and Food Sciences of Universities is suggested. A step in this direction has been taken by Sikkim University where a course microbiology on traditional ("ethnic") fermented foods and beverages of the world has been introduced at graduate level.

- **Restoration of "ethno-microbiology" practices in the Himalayas by women**

Some villages in the Himalayas, in the Darjeeling hills, Sikkim and East Nepal, involve traditional microbiological systems for *marcha*-making, and can be considered as centres of microbial and genetic resources. During a survey (Tamang, 2010) these villages have been identified as locations of possible microbial resources for traditional ("ethnic") alcohol production. By traditional procedures, these Himalayan women have been sub-culturing and maintaining a consortium of functional microorganisms for alcohol production in the form of dry ball-flatted cake-like starters called *marcha* (see also III. 4) or *phab* for more than 1500 years (Tamang, 2010). More information on these traditional starters and the locations of production is provided in the Annex 2. It has been recommended by Tamang (2010) that these villages, together with the traditional processes, should be declared as cultural heritage.

- **Traditional technology for making *Kinema***

Thanks to general demand, there is a good market for *kinema*, a traditional fermented soybean food in India, Nepal and Bhutan, similar to other *Bacillus*-fermented soybean foods of Asia (see Table II in Annex 1 on legume fermentations). Even when it provides a reliable source of income to many rural women, *kinema* processing has not been included yet in the loan-scheme of public sector banks or financial institutions, neither incorporated in the rural development programme and cottage-level industry scheme of any local government in the Eastern Himalayas.

- **Medicinal Aspects**

Thanks to the therapeutic values found in the traditional dietary pattern of the Himalayas typically, additional medicines or supplementary drugs are usually not required. Therapeutic effects are related to antioxidant, antimicrobial, probiotic and hypocholesterolaemic properties, in addition to the availability of essential amino acids, bio-nutrients, and some beneficial bio-active compounds (Tamang, 2007). More information is provided in the Annex.

- **Commercialisation through Ethnic Food Tourism**

Occasional visits by tourists to new places and countries are becoming popular all over the world. Some hill resorts in the Himalayas are becoming increasingly important as tourist destinations year by year. Among the major components of food tourism are the uniqueness of a food product, consumer behaviour, and traditional food production sites relevant to the local market potential. Moreover, traditional food is becoming increasingly attractive in destination marketing (Hall *et al*., 2003). The Himalayan traditional (ethnic) foods can be diversified into more presentable forms to attract the tourists to enjoy and experience their aroma, flavour and texture based on unique recipes. Promotion of food tourism will stimulate the opening of more ethnic food restaurants, food huts and stalls, which would focus on traditional utensils, kitchen wares, traditional culinary and customs and advocate the importance of ethnic values of the fermented foods. More information on this case study is provided in the Annex.

- ***Examples within the African context, based on collaboration with European partners and support by the EU***

Involvement of the EU in developmental projects and capacity building in Africa

An impressive number of EU-FP7 projects have focused on cooperation with Sub-Saharan Africa with a strong emphasis on capacity building and "North-South" exchange. Several of these projects may indeed serve as excellent examples of successful collaborative activities towards improving the useful exploitation of microorganisms in food processing.

Numerous bacterial and yeasts strains involved in the various fermentations, were isolated, identified and characterised, with respect to their functional properties and potential for technical applications. These strains were deposited in institutional culture collections. With a few exceptions, it is not certain whether means and ways have been found to maintain these collections and preserve the strains for long-term storage. They comprise a huge potential as genetic and biological resources for future applications. A few strains have been submitted to national culture collections (e.g. the DSMZ in Germany).

A number of EU projects funded under the Directorate-General for Health and Consumers (DG-SANCO), and supported by the World Association of Industrial and Technological Research Organizations (WAITRO) in joint actions, may serve as examples of successful collaboration between two European {Germany (DE) and Denmark (DK)} and several African partners. These projects focused at capacity building in research projects dealing with food fermentations of different kinds. The DANIDA (Danish International Development Agency) program has also supported similar activities in West African countries.

Some of the major outcomes of these projects include:

- The upgrading of several laboratories dealing in analyses and applied research in food microbiology and food hygiene.

- Training scientists from partner countries in well equipped European laboratories, and involving them in joint research projects.

- Opportunities for higher education comprised Master's and doctorate studies in Europe; in some instances, part of the work was conducted in laboratories of the African partners.

- Manual and guidelines ere developed and issued for small-scale food processing and for standardised procedures with regard to traditional fermentations, GMP and quality control.

- In search of a fermentative means for detoxification of maize, frequently contaminated with AFB1, hundreds of LAB strains were screened for AFB1 degradation, but with only with some strains showed ability to adsorb AFB1 from the medium, and thereby reducing the concentration of the mycotoxin. Among environmental (non-lactic) isolates from polluted soil, strains were found to be able to degrade AFB1, one of which was identified as a new species, *Mycobacterium fluoranthenivorans* (Hormisch *et al*., 2004). Other positive strains were identified as *Rhodococcus erythropolis* and *Nocardia* spp. (representatives of the *Actinobacteria*), and were found to completely degrade AFB1 (see Fig. 4) without any toxic or chemically detectable residues (Teniola *et al*., 2005; Alberts *et al*., 2006).

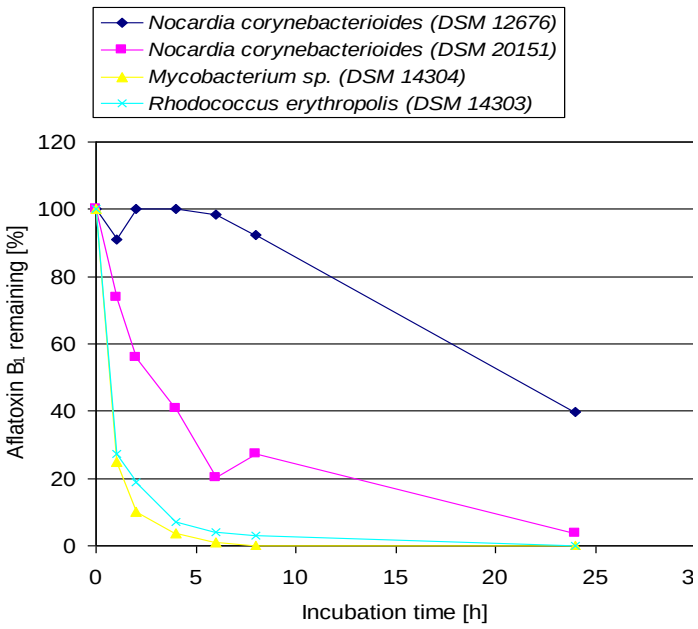

Figure 4a. Aflatoxin B_1 degradation at different incubation times using four (4) bacteria isolates cell free extracts obtained by French press method

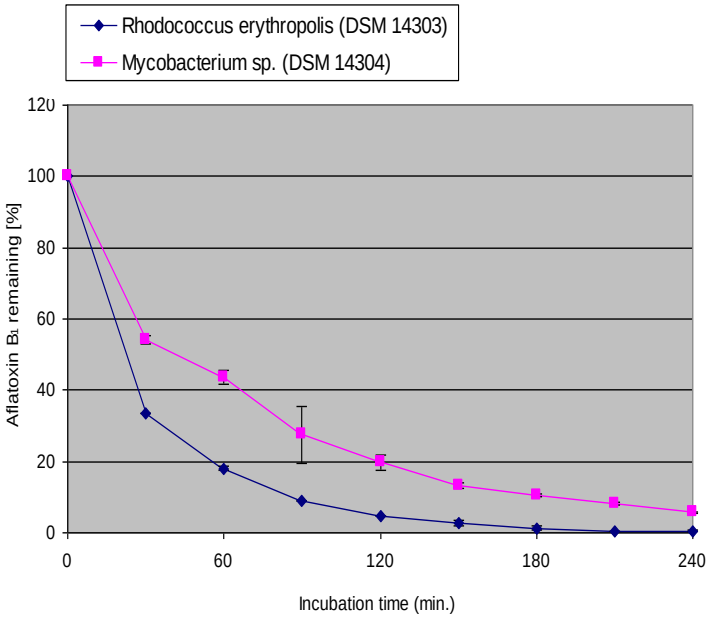

Figure 4b. Aflatoxin B_1 degradation at short incubation time intervals using cell free extracts of *Rhodococcus erythropolis* DSM 14303 and *Mycobactrium* sp. DSM 14304 obtained by French press method

Figure 4 (a and b). Aflatoxin B_1 degradation by cell-free extracts of different species of the *Actinobacteria*, showing complete breakdown of the AFB1 molecule (source: Teniola *et al*., 2005).

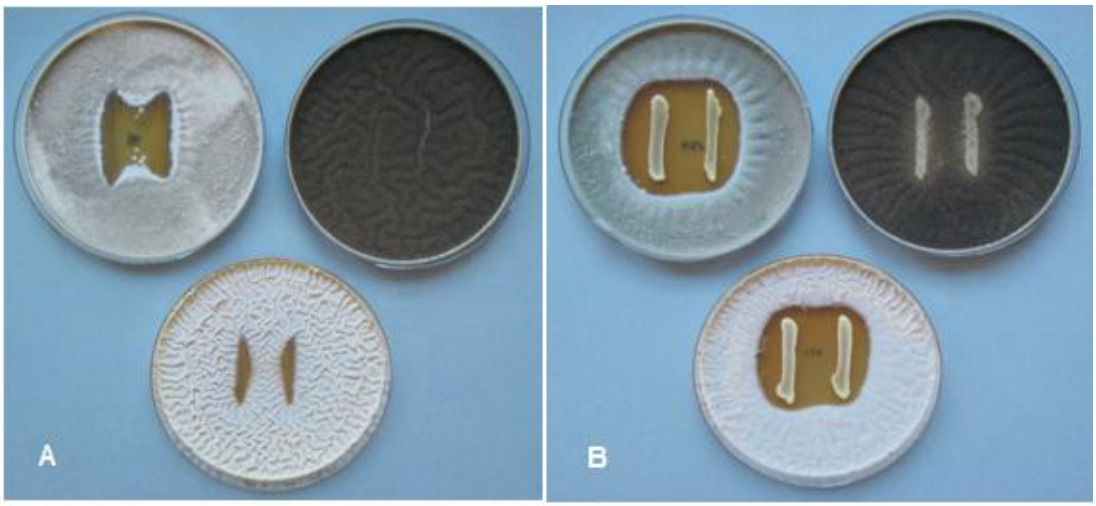

Figure 5. Inhibition of fungal strains isolated from coffee samples by *Lactobacillus* sp. BFE 6824 (A) and *L. plantarum* BFE 6862 (B), isolated from coffee fermentation. Fungal cultures: *A. ochraceus* BFE 686 (top left), *A. niger* BFE 702 (top right) and *A. ochraceus* BFE 722 (centre) (source: Färber and Holzapfel, unpublished results).

- Ochratoxin A (OTA) contamination of coffee is a serious problem hampering coffee exports from East African countries. Origin and mechanisms of OTA production in the coffee production chain have been identified in relation to the environmental and climatic conditions. Lactic acid fermentation dominates the post-harvest processing of Arabica coffee beans, and has been found to be an important quality-related factor. A few LAB strains associated with this fermentation showed ability to inhibit mycotoxinogenic moulds (see Fig. 6). In addition, a GMP manual has been developed.

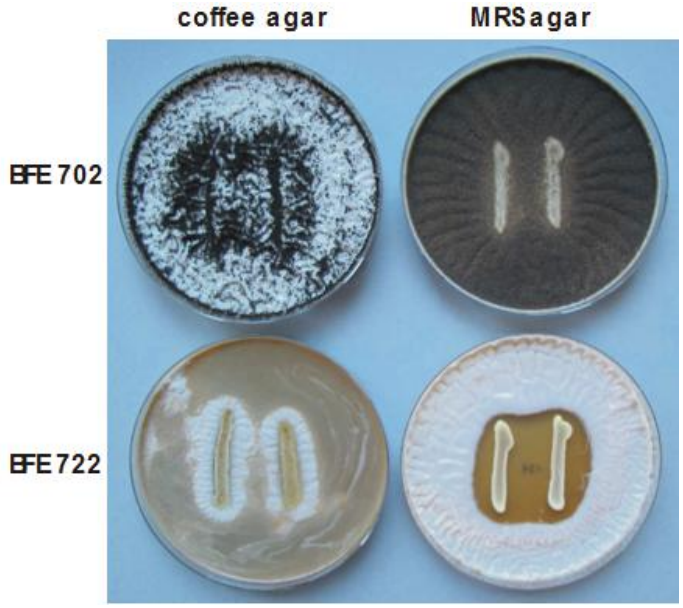

Figure 6. Comparison of the inhibitory activity of *Lb. plantarum* BFE 6862, isolated from coffee, during growth on MRS and coffee agar. Fungal test cultures of OTA producers: *Asp. niger* BFE 702 and *Asp. ochraceus* BFE 722 (source: Färber and Holzapfel, unpublished results).

- Biochemical and molecular markers were developed as indices for improving quality assurance in the primary processing of cocoa in West Africa, and also for this process a GMP Manual was developed.

- In a project conducted jointly in West and East Africa, starter cultures were developed for small-scale commercial fermentation of cassava towards Gari production (Hanak *et al*., 2008). Improvement of the nutritional status of Gari was achieved by fortification with soybean, palm oil and coconut milk. Two HACCP Manuals and 2 pilot plants (in Benin and Kenya) were developed. The starter cultures developed for cassava fermentation were standardised for the purpose of gari production (Kostinek *et al*., 2005; Yao *et al*., 2008; Edward *et al*., 2011). Large scale, concentrated starter cultures were pepared in a biotechnology plant by the Belgian partner (Univeristy of Liége), and applied for fermentation, both in Benin and in a newly established (womens' enterprise) pilot plant at the coast of Kenya. By this study, the following was achieved:

* Strains of the *Lb. plantarum*-group were effective in cassava fermentation and for reduction of the linamarin content.

* Establishment of a pilot plant for cassava fermentation and gari production in Benin in support of a womens' enterprise group (initiated by Prof. Egounlety, Cotonou), Benin (see Figure 7).

* Upgrading of cassava was achieved by addition of soya protein and palm oil.

* Starter cultures with excellent activity could be made available at low cost. Overall, the study showed that the starter strains could be easily and economically produced, thereby serving as a "model" for future starter culture development and application in a traditional fermentation set-up.

* "Technology transfer" for cassava fermentation and gari production in Kenya could be successfully achieved, and by establishing a womens'enterprise group near the Kenyan coast.

Figure 7. Women's enterprise group for cassava fermentation and production of fortified gari, near Cotonou, Benin. Starter cultures were developed and produced economically, for application as concentrate for cassava fermentation (courtesy: Mr. Moses Mengu).

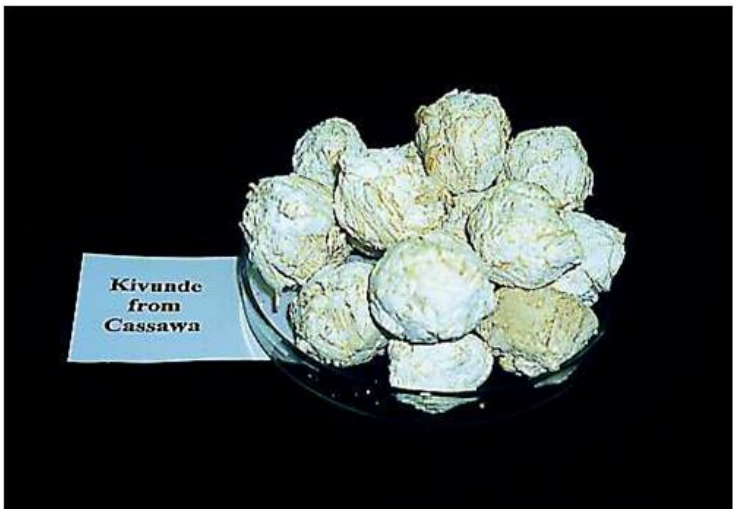

Figure 8. "*Kivunde*" produced by fermenting cassava with starter cultures (*Lb. plantarum* and *Lb. pentosus*) within the project "**Capability building for research and development in quality assurance and fermentation technology for traditional African fermented foods**" (EU Program: STD 3, DG XII/B).

Numerous other examples of successful research collaboration between partners from industrialised countries and scientists in developing regions, funded and supported by various national and international development organisations and agencies, can be mentioned. Put together, an impressive amount of R&D and capacity building activities in developing regions are ongoing thanks to the practical and financial support by different programs and at different levels of cooperation and coordination. A critical issue, however, remains the coordination, interaction, information sharing, and networking and exchange among these projects and activities. It has been realised that a more impressive impact may be achieved by aligning the research institutions "with African priorities and agendas". For this purpose the FARA Secretariat (http://www.fara-africa.org), EFARD (http://www.efard.eu), NATURA and the European Commission have agreed to coordinate activities around their research institutions. This resulted in the establishment of PAEPARD (Platform for African-European Partnership on Agricultural Research for Development) (http://www.fara-africa.org/our-projects/paepard/). PAEPARD is a sustained partnership for mobilisation of resources for priority projects that "combine African and European institutional and financial resources for mutually advantageous projects". FARA, (Forum for Agricultural Research in Africa) in collaboration with African research organisations, the CGIAR Consortium, and the International Food Policy Research Institute (IFPRI), is currently coordinating a series of technical regional workshops. The aim of these workshops is to enhance agricultural productivity through effective delivery of agricultural research, extension and education.

II.3.1.2. Impeding factors in the use of microorganisms in food processing

- Intellectual property rights (IPR) may be difficult to claim in view of the fact that fermentation processes in developing countries are based on traditional knowledge and skills. Claims of ownership of the traditional knowledge have to be supported by enhanced technical and scientific information on the craft and processing steps for indigenous fermented foods. The result was that individuals failed to realise the benefits of industrialisation of some traditional fermented foods. It is therefore important to place greater emphasis on issues relevant to IPR and on the precise characterisation of microbial strains involved in traditional fermentation processes. The importance of IPR education for local scientists, and the role of national governments for putting in place the required infrastructure for IPR for facilitating the process, has been adequately dealt with elsewhere (FAO, 2010).

- Regulations and procedures for approval of "novel" strains for applications in food biotechnology (referring to health claims, and safety). The recent restrictions imposed by EFSA on the use of health claims for probiotic strains is one example. Following initial rejection, 74 resubmitted probiotic health claims were all turned down again by the responsible EFSA panel. Some were initially rejected because of inadequate strain characterisation. For example, in the case of *Lactobacillus casei* DG CNCM I-1572, the Panel's view was that a cause and effect relationship has not been established between its consumption and the claimed decrease in potentially pathogenic gastrointestinal microorganisms (http://www.efsa.europa.eu/en/efsajournal/pub/2723.htm, *retrieved 29.09.2012*). In most cases, EFSA's opinion was that the claims were not justified by the evidence presented. EFSA concluded on *Saccharomyces cerevisiae var. boulardii* "*that a cause and effect relationship has not been established between the consumption of Saccharomyces cerevisiae var. boulardii CNCM I-1079 and defence against pathogenic gastro-intestinal microorganisms*". EFSA also considered that there was a lack of a proven cause and (beneficial physiological) effect relationship between the consumption of a combination of *Propionibacterium freudenreichii* SI 41 and *Propionibacterium freudenreichii* SI 26, related to increasing numbers of gastro-intestinal microorganisms (http://www.ingredientsnetwork.com/news-content/full/efsa-confirms-negative-opinion-on-74-probiotic-claims, retrieved 29.09.2012).

II.3.2. Ways to address current challenges and suggestions on how to make optimal use of existing opportunities

- Searching for "new" strains from traditional food fermentations and various ecological niches.
- Developing improved selection techniques and rapid identification of key properties of newly isolated strains.
- Direct identification of desired functional genes using up-to date PCR approaches,
- Improved knowledge and deeper understanding of the key technical and physiological properties of a microbial strain, including its exact taxonomy and, where possible, information on key functional genes and their stability.
- Use of modern approaches (metabolomics, proteomics, mathematical modelling) for studying the behaviour of a strain under processing and relevant environmental conditions; this should also include knowledge on process-related stress-response factors which may have an impact on the performance of the strain, and also knowledge on its "housekeeping" and key functional genes.
- Making better use of recent progresses in basic scientific knowledge. Example: Progress in knowledge on the **human gut microbiome**, thanks, amongst others, to the Human Microbiome Project (HMP) (Methé *et al.*, 2012; Huttenhower *et al*., 2012). Thousands of different microbial species have been mapped, thereby opening new areas of research and of deeper understanding of the diversity (Li *et al*., 2012), and the role and importance of the microbiota associated with the human GIT. It is staggering to consider that about 23 000 human genomes have been identified, but that, by contrast, eight million different kinds of unique microbial genes are associated with the human body (http://www.cbc.ca/news/health/story/2012/06/13/human-microbiome-project.html).
- With regard to constraints imposed by EFSA on health related claims for probiotics (discussed under **2.3.1.2**), the approach of the Japanese government by introducing an approval process for functional foods, called "Foods for Specified Health Use" (FOSHU) in the 1980s, may serve as classical example worthwhile of pursuing. With only one formal approval by EFSA of a probiotic health claim (alleviation of lactose tolerance) as from December 2012, around

955 foods (including probiotics) have been approved for FOSHU by the Consumer Affairs Agency of the Japanese Ministry of Health, Labour and Welfare by 2011. The approval process involves a detailed review process of a dossier in which scientific evidence is supplied for each application. This well-established procedure for establishing Nutrition Labelling Standards in Japan is conducted along with participation by the CODEX Committees on Food Labelling and on Nutrition and Foods for Special Dietary Uses (Codex Alimentarius). All-over, these activities are coordinated by the WHO Global Strategy on Diet, Physical Activity, and Health, and are an example of successful controlled regulation of the promotion of "health foods". Moreover, the HMP project (discussed before) produces an immensive amount of data on the gut microbiome, and may link specific genes of beneficial strains with their functional performance and health related properties. By this development, a solid scientific basis is established which may assist future decision-making on health related claims.

- Continued search for new strains for use as starter cultures for cheese; in particular phage-„unrelated" strains, also with good technical properties, are constantly required, also for enabling new culture regimes in dairy industries. Traditional fermented foods constitute a potentially valuable source of such „new" strains.

- Promotion of small-scale enterprises, and support/back-up for "risk-taking" steps towards scaling up and standardisation of processing operations. Examples of successful womens' initiatives may serve as models as catalysts for economic change, and as encouragement for training and organising of entrepreneurs. An excellent example is the successful small-scale (non-household level) production of kenkey by a womens' intiative in Accra, Ghana (see Figure 9).

- Identification of local or regional leaders with innovative ideas, e.g., for developing new products and initiating activities based on traditional resources, skills and practices.

Figure 9. After soaking of maize and wet milling, the dough is fermented ("*aflata*") for making *kenkey*. Left: traditional, household scale; right: small (women's) enterprise in Accra, Ghana (photographs: Holzapfel).

- Studying synergistic and "implicit" properties of biodiverse culture communities associated with traditional fermented foods valued for excellent product and sensory features.

- Developing mathematical models for predicting the behaviour of a complex microbial community during different phases of fermentation, and taking into account extrinsic (temperature, atmosphere/climate) and intrinsic (pH, Eh, a_w, nutrients) factors.

- Bio-preservation is an attractive alternative to chemical and physical preservation. Food fermentations offer excellent models for studying the basic principles and mechanisms of bio-preservation, and traditional fermentations may be valuable resouces of new strains for bio-preservation. Several LAB strains are commercially distributed as protective cultures for preservation and safeguarding of foods against different pathogens and for various food substrates.

- Preparing an inventory of strains related to traditional food processing, and deposited in institutional, "non-formal" and private culture collections.

- Establishing:

  - ✓ a "**Global Collection of Microbial Strains for Small-scale Food Fermentation**", and

  - ✓ **Regional collections** for depositing strains typical of an area or region, and taking into account specificities and peculiarities (e.g., differences between traditional East and West African fermentations).

- Making available both information and microbial strains (at low cost) deposited in numerous institutional and laboratory culture collections.

- BCCM launched the concerted action **MOSAICC** (**M**icro-**O**rganisms **S**ustainable use and **A**ccess regulation **I**nternational **C**ode of **C**onduct) with the support of the Directorate General XII for Science, Research and Development of the European Commission. The project started in September 1997 for duration of 21 months and involved the following twelve partners: **OECD, IUCN, The Royal Botanic Gardens KEW, Novo Nordisk A/S, IMI, WFCC, IPM, CCT, INBio, UICC, ARC and BCCM** (see list of Acronyms and Abbreviations).

MOSAICC is a voluntary Code of Conduct, a tool to support the implementation of the Convention on Biological Diversity (CBD, Rio de Janeiro 5 June 1992) at the microbial level, in accordance with other relevant rules of international and national laws (http://bccm.belspo.be/projects/mosaicc/). The impression is that the numerous rules and stipulations might constitute extremely complicated hurdles for small-scale enterprises wishing to make use of the opportunities offered.

Opportunities

Various national and international organisations, including NGO's (e.g., Oxfam International, FIAN, FHI, CARE, FAM, CRS, World Vision, Caritas) are committed to the promotion of food safety and security in developing nations. These NGO's as potential resources of support have not been fully exploited yet, partly due to shortcomings in communication, information dissemination, and/or lack of coordination.

Food programmes for developing nations offer numerous opportunities of support via research and development projects. However, the potential for leadership and management are two key factors towards the successful initiation and preparation of a new proposal, followed by effectively coordinating and assembling a consortium of selected team members. Several private companies or institutes have been established (e.g., in Brussels) to support scientists in the preparation of new project proposals.

Among the numerous opportunities offered by international organisations and joint programs, some examples of opportunities offered and decisions taken on specific support are given below:

- The International Union of Food Science and Technology (IUFoST) released on November 2010 an outline plan of work for the global food science and technology community. Known as the"

Cape Town Declaration", it states the right of every individual to have access to adequate and safe food (http://iufost.org/iufost-introduces-outline-plan-work-global-food-science-community) by, *inter alia*:

- adaptation and improvement of traditional foods and processes, while respecting the traditional, ethical, cultural and religious aspects involved;
- development of food materials with improved functionality;
- education in nutrition, food science and technology at all levels.

- Strains with excellent functional (probiotic) properties have been reported for a range of traditional foods, both from animal (Mathara *et al*., 2004, 2008a,b), and plant origin (Todorov *et a*l., 2007; Tamang *et al*., 2009a; Lee *et al*., 2011), and also of the fermented plant product (Kim et al., 2004; Sheo and Seo, 2004). These strains, mainly deposited in small institutional cultural collections, constitute a valuable potential for exploitation and development of functional fermented foods.

- Challenges and opportunities for new developments in the functional food market are indicated by the fact that probiotics were the 3rd fastest growing supplement globally in 2011 (Hudson, 2012).

- "Digestive health beverages", expected to take a larger proportion of the future market (identified as "expansion category"), already represented a market volume of US$ 890 million in 2010, and comprising probiotic and fibre juices (Hudson, 2012), and with several national and international consortia entering this market.

- Directions of future developments will primarily be focused on general well-being, with short-term growth expected to be driven by issues such as cardiovascular health, bone and joint health, respiratory health, and immune support (Hudson, 2012). Research on probiotics, including double-blind, placebo-controlled clinical studies in recent years have supported a number of health-related claims, including the health issues mentioned before.

- Manufacturing and characterising the microbiological, textural, nutritional and sensory properties of new food mixtures with improved functionality, e.g., vegetable yogurt-like beverages made of mixed cereals (rice, barley, emmer and oat) and soy flours and concentrated red grape must, was suggested by Coda *et al*. (2012).

- New techniques are being studied and adopted for developing and promoting functional foods with a greater shelf-life, with strains adapted to different food matrices, and with new "multi-functional" strains that are metabolically active in the food substrate, and, in addition, having antimicrobial properties for extending shelf-life of the product. New food matrices as carriers of probiotic strains may include meat, cheese, fruit juices, breakfast cereals, ice cream and chocolate.

II.4. Impact of climate change on the sustainable use of microorganisms in food processing

II.4.1. Past, current and potential future impacts of climate change on the use of microorganisms in food processing.

Whatever the case on global temperatures may be, numerous indicators underline regional climate changes on all continents. The sad fact is that desertification is increasing at an alarming rate, threatening the existence and traditional lifestyle of around 2 billion people. Multiple factors, as shown for the Sub-Saharan region, are responsible for this development, some of which are extremely difficult to control (IFFN, 2007). The result is a continuing movement of people from traditional urban areas, mainly to larger cities. This has a strong impact on food consumption patterns, concomitantly

leading to a shift from traditional foods towards "Westernised" diets. The urbanisation process will inevitably result in a change in lifestyle and dietary patterns, frequently leading to a loss of knowledge and experience in traditional skills in food fermentation. Also, the benefits of traditional fermented foods, as they were consumed through generations, may not be experienced anymore by urbanised communities. Potential genetic resources, harboured in diverse microorganisms associated with these foods, may be permanently lost when traditional fermentations are given up.

The influence of climate change on microbes and their response, e.g., by adaptation, is an important issue. Trait-based frameworks are being developed for predicting microbial adaptation to climate change, and concomitantly, the impact on the functioning of biogeochemical processes in aquatic and terrestrial ecosystems. As microbial communities are typically not optimally adapted to their local environment, it may occur that the temperature optima for growth and enzyme activity determined under laboratory conditions may be higher than *in situ* temperatures in their environment (Wallenstein and Hall, 2011). Models on this basis, i.e., also taking into account "natural" conditions, may be helpful to predict possible changes in microbial interactions and behaviour in food substrates.

Within the controlled set-up of modern laboratories, the impact of intrinsic (e.g., substrate composition, pH, a_w, redox potential), and extrinsic factors (e.g., temperature, aerobic vs. anaerobic conditions, humidity) can be studied for an organism, and the optimal conditions for its growth and metabolic activities determined. The diversity among microorganisms with regard to their optimum growth range as influenced by temperature, pH, gaseous atmosphere, is well documented. These data are mainly based on well-defined laboratory conditions, but they may serve as basis for developing predictive models of simulated microbial behaviour (Membré *et al*., 2005; McMeekin *et al*., 2008), including for fermentative LAB such as *Lb. curvatus* (Wijtzes *et al*., 1995). A novel model class prototype explicitly incorporates nutrient exhaustion and/or metabolic waste product effects. Constituting an elementary building block, it can be extended in a natural way towards microbial interactions in co-cultures (mediated by metabolic products) and microbial growth in structured foods with differences in, e.g., local substrate concentrations (Van Impe *et al.*, 2005).

Moving into the era of systems biology, with increasing availability of physiological and molecular information for incorporation into models, predictive microbiologists will be faced with new challenges of handling experiments and resulting data. It is expected that interaction between microbiologists and mathematicians may result in the development of new models for describing the microbial role in ecosystems other than food (McMeekin *et al*., 2008).

In an industrial fermentation process, all influencing factors (temperature, pH, aeration, substrate, etc.) are precisely controlled, and the use and handling of defined starter cultures is well established. These processes can be monitored and technically controlled independently of the climate, using computerised systems, also relying on the predictable behaviour and output of a starter culture. The behaviour and performance of a defined starter culture can be predicted by using mathematical models, as mentioned before. By contrast, as far as is known, models are not available yet for predicting climate related impact on microbial behaviour, under artisanal and most other small-scale conditions. These fermentations may therefore be influenced in an unpredictable manner by temperature and other climate related factors such as humidity and availability of potable water. Estimations of changes in a complex, "multiple strain" fermentation can, however, be made with reference to some recent laboratory studies on succession and domination in a mixed culture fermentation. Information obtained by pure culture (single strain) studies, will not reflect the impact of all relevant factors on a mixed strain culture (e.g., competition, antagonism, mutualism, etc.), composed of diverse strains and species. Intra- and interspecies diversity is discussed in Section 2.2 ("Microbial Diversity in Food Processing").

Climate change may have a significant influence on post-harvest scenarios related to mycotoxin production (Paterson and Lima, 2010). However, only sparse information, including simulation models, is available on the effect of climate change on pre- and post-harvest conditions, and the occurrence of mycotoxins in foods (Miraglia *et al.*, 2009). From knowledge of fungal metabolism, it

can be expected that a temperature increase in cool or temperate climates, will increase the risk of aflatoxin formation, a mycotoxin not associated with crops indigenous to Northern Europe and the moderate central European regions. The currently moderate to cool regions may become liable to temperate problems with ochratoxin A (OTA) production by *Aspergillus ochraceus* (hitherto associated with tropical countries), in addition to the *Penicillium* species, *P. verrucosum* and *P. nordicum*, typical of moderate climate regions and respectively associated with OTA formation in cereals and meat products. *Aspergillus ochraceus* has an optimum temperature of 25-30 °C for OTA production, as compared to 25 °C for *P. verrucosum* (Paterson and Lima, 2010). In a study on the influence of temperature on OTA producing fungi, optimal OTA production by *Asp. niger* var. *niger* BFE 632, isolated from coffee fermentation, occurred at lower temperatures (23-25 °C) than the control strain, *Asp. ochraceus* CBS 589.68 (30 °C), but was within the range of *Pen. nordicum* and *Pen. verrucosum* (Färber and Holzapfel, unpubl. results) (see Figure 10).

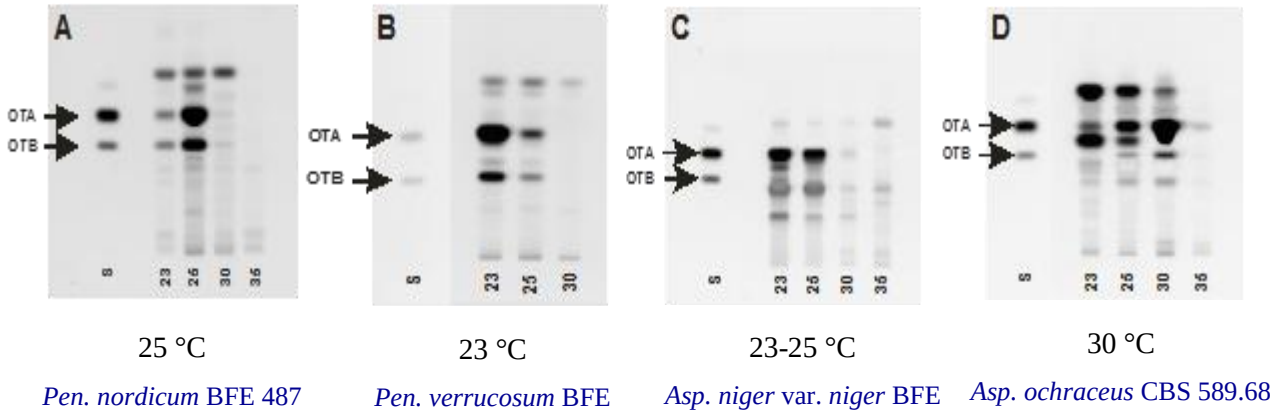

Figure 10. Temperature dependence of OTA biosynthesis - examples (growth in YES - Medium for 9 days) (Färber & Holzapfel, unpublished results)

Maize (corn) is a staple for many millions of people throughout Africa, Asia and the Americas. It serves also as an important raw material for numerous lactic fermentations in Africa and Central America (compare also Table III in Annex 1). However, this crop is particularly vulnerable to influences of climate, thereby increasing the risk of mycotoxin formation, which will be carried over via fermentation to the final product. Therefore, mycotoxin management technologies are urgently required for providing safe raw materials to local markets for fermentation. Detoxification, e.g., as part of the fermentation process, and shifting of cropping patterns may offer potential solutions (Cotty and Jaime-Garcia, 2007). The issue of detoxification is discussed in Section 4. (b).

Among all factors related to climate change, temperature would probably have the greatest impact on the behaviour of microorganisms in food fermentations. With a change in temperature, expression of key functional genes may be influenced, thereby altering the formation of microbial metabolites. Ultimately, the balance of the process will be shifted. Each microorganism has an encoded ability to produce specific metabolites. Different genes are grouped in operons and are frequently expressed as groups. However, these processes are highly regulated and no strain will express all these genes simultaneously (Todorov, 2009), but in reality only express those that are required under a given set of conditions (physico-chemical and biological factors) at a specific moment and time. Activation and expression of different genes may depend on the presence of certain metabolites, resulting in "feed-back" response and even inhibition, but are also determined by changes in temperature.

The following examples may give some insight into the influence of key climatic factors such as temperature, on (e.g.) population shift, and the formation of some primary (e.g., lactic acid) and secondary (e.g., bacteriocins) metabolites.

Gänzle *et al*. (1998) modelled the growth of two strains of *Lb. sanfranciscensis* and of *Cand. milleri* in response to process parameters of sourdough fermentation, taking into account the combined effects of pH, temperature, salt concentration, and accumulation of metabolic end products (lactate, acetate and ethanol). The purpose was to identify the key factors contributing to the stable association of lactobacilli and yeasts. Interestingly, *Cand. milleri* had a lower optimum growth temperature (27 °C) than the lactobacilli. Except for sensitivity to 150 mmol of undissociated acetic acid per liter, the growth of the yeast was not affected by pH in the range of 3.5 to 7, and up to 8% NaCl. It may thus be assumed that the difference in optimum temperature would be a key factor influencing the balance between the lactobacilli and yeasts in "spontaneous" traditional sourdough fermentations. Depending on the yeast species and strain, the lactobacilli may be favoured in situations of drastic rises in ambient temperatures.

Monitoring the growth of *Lactobacillus* species during rye flour fermentation, Müller *et al*. (2000; 2001) investigated the effect of external factors under the controlled conditions of a lab-scale process, with special focus on temperature. Fermentations were conducted at 34.8, 40.8 and 46.8 °C, resulted in microbial communities identical to those found in an industrial scale. It is of significance that the qualitative composition of the microbiota was not affected by the temperature, with *Lb. amylovorus* (homofermentative) as the dominant species. However, with increasing fermentation time, a shift towards predomination of heterofermentative lactobacilli was documented. Increasing temperature also resulted in a reduction of the percentage of homofermentative lactobacilli.

In another study on sourdough fermentation, Messens *et al.* (2002) investigated the biokinetics of cell growth of *Lb. amylovorus* DCE 471 and its bacteriocin production as a function of temperatures in the range 28 ° to 44 °C, and pH values (pH 4.2 to 6.4), characteristic of this fermentation. A predictive model describing the influence of temperature and pH on microbial behaviour was developed and successfully validated.

Studying the production of an antimicrobial peptide (nisin) by a strain of *Lact. lactis* isolated from goat milk (Furtado *et al*., in preparation) showed that these antimicrobial peptides are expressed only at 30 °C and not at 37 °C. It was also found that the immunity protein, located in the same operon ash the structural gene of the antimicrobial peptides, was expressed at 30 °C. However, the immunity protein was silenced at 37 °C, indicating that the structural gene, as part of the same reading frame of the antimicrobial peptides, has not been expressed at this temperature.

Other antimicrobial peptides such as those produced by *Lb. plantarum* LP08AD (isolated from donkey milk), *Ent. faecium* ST5HA (isolated from smoked salmon), *Ent. mundtii* ST4SA (isolated from soya beans), *Ped. pentosaceus* HA-6111-2 and HA-5692-3 (isolated from Alheira, a traditional fermented sausage from Portugal), *Lb. plantarum* ST202Ch and ST216, *Ent. faecium* ST211Ch, *Lb. sakei* ST22Ch, ST153Ch and ST154Ch (isolated from fermented smoked and cured meat products from Northern Portugal), *Ent. faecium* AQ71 (isolated from Azerbaijani Motal cheese), *Ent. faecium* SD1, SD2, SD3and SD4 (isolated from Sardinian goat's milk), various strains of *Lactobacillus* spp., *Enterococcus* spp., *Leuconostoc* spp. and *Lactococcus* spp. (isolated from boza, a fermented cereal based beverage from Balkan peninsula) (Ahmadova *et al.*, 2012; Albano *et al.*, 2007; Muria *et al.*, in final preparation; Schirru *et al.*, 2011; Todorov and Dicks, 2005; Todorov and Dicks, 2009; Todorov, 2010; Todorov *et al*., 2012; Todorov *et al*., 2010a,b; 2013; Von Mollendorff *et al*., 2006) have been expressed at different levels when the bacterial strains were cultured at different temperatures. These data strongly underline the influence of temperature on the features and functionality of the final product. Active involvement of these strains in natural processes for the manufacture of fermented milk products (cheese, traditional and probiotic yoghurts), boza, smoked salmon or meat products, will determine specific metabolic activities influencing, e.g., sensory attributes. These may include the production of lactic acid and diacetyl, the production of proteolytic enzymes resulting in degradation of some proteins (thereby influencing the texture but possibly also the allergenicity of the final product).

The production of antimicrobial peptides such as bacteriocins by LAB may have a significant influence on the safety of the final product. Scientific observations suggest that LAB are involved in the reduction of pathogenic bacteria in the food products based on antimicrobial metabolites. Apart from organic acids (mainly lactic acid but also acetic acid from heterofermentation), various other microbial metabolites such hydrogen peroxide, low molecular weight metabolites, and antimicrobial proteins are associated with these antagonistic effects (Holzapfel *et al.*, 1995). The primary metabolites (e.g., organic acids, hydrogen peroxide or carbon dioxide), are constantly expressed, albeit at varying rates according to the growth conditions, while the formation of secondary metabolites such as antimicrobial peptides and bacteriocins, antibiotics, and several toxins may be determined by the specific temperature; moreover, they are typically produced during the later growth stages, and in response to particular culture conditions.

It should be underlined that the presently observed shifts in the climate towards desertification have a catastrophic impact on people already living in poverty-stricken regions. Changes in climatic conditions may influence the microbial ecology in all natural habitats. A rise in temperature may result in a shift in the microbial domination, and in some cases, may accelerate the fermentation in a favourable manner. However, the typical metabolic pattern of the traditional fermentation may be changed, resulting in a negative effect on the typical sensory features of the product. A more devastating effect of desertification is an increasing water shortage, thereby limiting the application of traditional artisanal fermentation practices. These changes will have a stronger influence on rural communities of already struggling communities in Africa, Asia and Latin America. By their lifestyle, these communities are more dependent on natural fermented food processes as compared to highly industrialised regions of North America, Europe, East Asia and Australia.

II.4.2. Can the use/application of microorganisms in food processing mitigate the effects of climate change in any way?

The global increase in the output of CO_2 has become an issue of major concern. The continued use of fossil fuels as major energy source puts a large amount of CO_2 into the atmosphere additional to that which is "recirculated" in terms of the carbon cycle. The latter refers to CO_2 production by natural processes such as mineralisation (decomposition of biomass from plants and animals), and by its capturing via photosynthetic activities of micro- (e.g., micro-algae, cyanobacteria), and macro-organisms (plants). Microbial degradation of plant matter into soil (frequently overlooked as environmental factor) releases estimated 55 billion tons a year of carbon dioxide, 8 times what humans are putting into the atmosphere through fossil fuel burning and deforestation (Zimmer, 2010).

The amount of CO_2 produced by food fermentations can therefore be considered negligible compared to the output from other sources. Research activities on the sequestration of CO_2 by photosynthetic microorganisms have been intensified as a means of reducing the CO_2 concentration in the atmosphere and, simultaneously, for the production of biofuels. Advantages of micro-algae as potential sources of bio-energy include their photosynthetic efficiency, faster growth rate, and their high biomass and oil productivity compared to oil crop plants, yielding up to 50% lipids in the dry biomass. Of high relevance is also that algae are not competing with land-based food plants (so-called "energy crops") as resources of bio-energy (Miao and Wu, 2006; Li *et al*., 2007; Halim *et al*., 2012).

Even the most beneficial fermentations will produce waste products. The composition and amount of these products will determine whether it will be considered as a waste product or by-product. Large and medium sized industries usually have the technological means and infrastructure for converting a waste material, either into a valuable by-product or for treating the material to reduce the Biological Oxygen Demand (BOD), and thus its pollution potential, thereby preventing its potentially harmful impact on the environment.

The waste management and processing of waste materials and/or by-products may have a decisive influence on the economics and feasibility of a fermentation process. Local municipalities or authorities require the quality waste-water to conform to regulations, otherwise resulting in heavy

penalities. Pretreatment of waste-water before entering the municipal sewage system may therefore be essential for a company. The organic load of waste-water can be calculated on the basis of those substances that can be oxidatively eliminated or transformed by microorganisms. BOD5 or the "biological or biochemical oxyen demand" refers to the amount of O_2 that is utilised within 5 days by the microorganisms present (as mg of O_2 utilised/l at 20°C; iodometric determination). Some considerations with economic impact for the treatment or further processing of by-products may include waste-water or sludge with a high load of organic material that may be converted in digesters or decomposing reactors to biogas (CO_2 und CH_4) – which, as "green-house" gases, may cause additional environmental pollution.

A typical example is the production of large volumes of whey during the manufacturing process of cheese. About nine liters of whey are produced for every kilogram of cheese, thereby yielding a large volume of a waste- or potentially valuable by-product. The cheese whey contains around 1 % of proteins (albumins and globulins) and 4 % of lactose, both of which can be considered as valuable by-products, should they be recovered from the whey in concentrated form. Other processes have been developed for the fermentative conversion of whey into lactic acid or single cell protein. In all these cases, the potential of pollution will be drastically reduced. The homofermentative conversion of the whey lactose to L(+) lactic acid by *Lb. casei* has been reported to result in a high lactose conversion of 95.6 % under optimised conditions (Panesar *et al*., 2010). An advantage of well-controlled biotechnical processes of this kind is not only the yield of a valuable end-product (in this case the "physiological" isomer of lactic acid), but also to prevent any significant CO_2 production by choosing a homofermentative LAB species.

Huge amounts of CO_2 are being produced during practically all industrial alcoholic fermentations resulting from the glycolysis metabolic pathway of the yeast, *Saccharomyces cerevisiae*, e.g., for the manufacture of beer and wine. Some factories make use of the CO_2 for carbonation of soft drinks, and for industrial utilisation as "dry ice".

These examples refer to industrial scale operations. When conducted by medium to large companies, the technical and infrastructural means usually exist to prevent or reduce pollution significantly.

Small scale processors, particularly those relying on traditional artisanal practices, are not equiped to handle waste products efficiently; both low-tech infrastructure and economic constraints will reduce their abilities for complying, e.g., to local municipal waste-water regulations. In rural areas of developing regions, restrictive regulations on pollution may probaby not be imposed. In terms of CO_2 and waste-water output (partly resulting in anaerobic degradation with the formation of methane), these probably have negligible impact on the climate. Obviously, pollution remains a serious problem in most developing regions.

Clostridium ljungdahlii, an anerobic bacterium, might be defined as a "multifermenter", being able to ferment everything from sugars to simple mixtures of carbon dioxide and hydrogen, and even „syngas" ($CO + H_2$), forming biofuel. According to Köpke *et al*. (2012), its synthesis capabilities from CO and CO_2 are not limited to biofuels, but can be expanded to virtually every compound for which biological pathways exist. It is not known whether it is adapted to the fermentation of food substrates, but the diverse metabolic abilities and adaptation patterns of bacteria might open new prospects of capturing CO_2 even in the context of food fermentation.

Considering that all food fermentation processes contribute only a minute fraction of the total daily greenhouse gas output, the consideration of alternatives may only in rare cases be feasible. Beer brewing and winemaking can only be conducted by fermentation; artificial processes using enzymatic conversions and chemical modification may not result in an acceptable product for consumers. Similar considerations will be valid for most traditional fermented foods, including sauerkraut (in Europe) and kimchi (in Korea). Interestingly, these two kinds of fermented vegetables are being produced on both industrial and household scales, and, in both cases, still by observing traditional practices and principles. The diverse species involved in these fermentations include both hetero- (CO_2 producing)

and homofermentative LAB. On the other hand, for preserving some vegetables such as cucumbers, marinating in an acid solution, usually vinegar, is commonly practised as alternative to (lactic) fermentation, but results in a product with different sensory qualities.

III. CURRENT STATUS AND TRENDS IN THE CONSERVATION OF MICROORGANISMS USED IN FOOD PROCESSING

III.1. Current conservation methods

Thousands of useful microorganisms constitute the basis of biotechnological processes. Parallel with the process of isolation, selection and genetic engineering a need arises for the preservation of strains, their vitality, specificity, activity, immunogenicity and other properties in laboratory conditions (Tsonka and Todor, 2005).

The primary objective of preserving and storing an organism is to maintain it in a viable state without morphological, physiological, or genetic change until it is required for future use. Ideally, complete viability and stability should be achieved, especially for important research and industrial isolates. However, even teaching or research collections may have to consider additional factors such as simplicity, availability, and cost. Preservation techniques range from continuous growth methods through to methods that reduce rates of metabolism to the ideal situation where metabolism is suspended (Smith *et al.*, 2001). Depending on the aims, the methods could be divided into:

- laboratories with short time between cultivations – from several months to years (sub-cultivation, storage under mineral oil, in water, by cooling to 4 – 8 °C or drying, etc.);
- long-term storage methods, by which the specimens could be preserved unchanged for decades (Tsonka and Todor, 2005). In general, long-term conservation of biological resources depends on rendering cell into a metabolically inactive state that ensures their long-term stability. This invariably depends on the removal of water, or making it unavailable for biological, and if possible chemical, activity. The most commonly employed approaches involve the use of freeze-drying or cryopreservation techniques (Day and Stacey, 2008).

III.1.1. Sub-cultivation

The sub-cultivation is a method of periodical cultivation on an agar nutrient medium. It is widely used and it is perhaps the oldest, simplest and most cost effective method for microorganism maintenance and preservation under laboratory and industrial conditions, especially if cultures are required frequently and quickly. The main principle in the cultivation is taking cell material from great amount of colonies. Using a single colony is not recommended because this increases the unwanted selection probability. Thus the control of the innate strain characteristics, activity change and vitality could be impossible. The choice of nutrient medium for strain cultivation is essential for the method application. Choosing correct nutrient compounds is the base of further preservation of taxonomical, morphological and biochemical culture properties. Refrigeration below room temperature is often used as it extends the subculture interval thereby reducing the number of transfers required as a result of the suppression of the metabolic rate. The regularity of the cultivation is different for the separate micro-organism groups and varies from 30 days to several years, at preservation temperature 3 – 5 °C. The average conservation longevity for yeasts is 1 to 3 months. Data exist that some bacteria are conserved for 5 to 12 months and microscopic fungi over 5 years. Available literature indicates that representatives of genus Streptomyces keep their vitality by this method for 26 years. Fungi are the longest preserved by sub-cultivation strains and have been kept since 1895 (Smith *et al.*, 2001; Tsonka and Todor, 2005).

III.1.2. Storage under mineral oils

This method is generally only used for yeasts and filamentous fungi but can be applied successfully to bacteria. The method essence is covering the well grown culture on liquid or agar nutrient medium with sterile non-toxic mineral oil. The aim is to limit the oxygen access that reduces the microorganisms' metabolism and growth, as well as to restrict the cell drying during preservation

in freezing conditions. Retrieval is relatively easy and involves the removal of a small section of the colony with a mounted needle. Excess oil is drained away and the inoculum streaked onto a suitable agar medium. The fungal mycelium can normally recover when it is re-isolated from the edge of the colony on the first agar plate and transferred to fresh media. According to some investigations the microorganisms' conservation period under vaselline oil without sub-cultivation is 1 to 12 years depending on their properties. Optimal and utmost time limits are established for cultivation of different taxonomical groups. The preservation period for bacteria from the genera *Azotobacter* and *Mycobacterium* is from 7 to 10 years, for *Bacillus* 8 to 12 years. During a 12 month preservation period of the genus *Lactobacillus* under vaseline oil, the titer decreased 2 – 3 orders, but there are some data for conservation of the genus for up to 6 years. Other bacteria need re-cultivation at intervals of 6 months to 1 year. *Acetobacter* and *Gluconobacter* have to undergo up to five passages for recovering of biochemical properties. Different yeast genera are studied and it is determined that the conservation period varied from 1 to 7 years (*Candida, Endomycetes, Hansenula, Saccharomyces, Schizosaccharomyces, Pichia*). A wide range of fungi survive this method, while *Saprolegniaceae* and other water moulds survive 12-30 months. Species of *Aspergillus* and *Penicillium* have remained viable for 40 years and some strains of *Phytophthora* and *Pythium* species have survived up to 40 years. Many cultures have been found to deteriorate under mineral oil and have to be transferred regularly to reduce this effect. However, organisms that are sensitive to other techniques can be stored successfully in oil, for example *Cercospora*, *Arthrobotrys*, *Colletotrichum*, *Conidiobolus*, *Corticium*, *Nodulisporium* and mycelial *Basidiomycetes* should be transferred every two years. The disadvantages of oil include the possibility of contamination by air-borne spores, retarded growth on retrieval, and continuous growth under adverse conditions that could have a selective influence. However, preservation under oil is recommended for storage of organisms in laboratories with limited resources and facilities. The advantages of oil storage are long viability of some specimens and survival of species that do not survive other preservation regimes. Additionally, mites do not cause infestations in oil cultures as they are unable to escape once they have entered the culture bottle (Smith *et al.*, 2001; Tsonka and Todor, 2005).

III 1.3. Water storage

Immersion in sterile water can be used to extend the life of an agar culture. The method is generally applied to fungi and can be achieved in many different ways. One simple way is to grow the fungus on an agar slope in a Universal bottle and then cover the agar surface with water. The shelf-life of fungi in water is variable but there are examples of phytopathogenic fungi successfully stored for ten years by this means. Strains of *Pythium* and *Phytophthora* stored in water for five years but only 58% of these remained viable. From 78 isolates, belonging to seven genera, preserved in water for 12 years, on resuscitation, 89.7 % of isolates were viable. Water storage did not significantly affect growth rate, viability or genetic stability in 155 isolates of *Basidiomycota* stored for 7 years.

The advantages of storage in water are the low cost and easy application. However, the length of storage is often limited and some fungi will not survive even short periods submerged. As with all methods that allow growth or metabolism during storage there are better methods and it is considered only to be useful for short-term preservation (2-5 years) and should be backed up by longer-term storage methods (Smith *et al.*, 2001).

III.1.4. Silica gel storage

The technique is relatively simple and involves the inoculation of a suspension of fungal propagules onto cold silica gel. The culture will then dehydrate to enable storage without growth or metabolism. Silica gel storage has a number of advantages; it is cheap, simple and does not require expensive apparatus. Sporulating fungi have been stored for 7-18 years in silica gel and appear to remain morphologically stable after resuscitation. Cultures are relatively stable, allowing a wide range of sporulating fungi (including representatives of the *Basidiomycota*) to be successfully preserved. Penetration by mites is unlikely, as they cannot survive the dry conditions encountered. Repeated inocula can be removed from a single bottle. There are some disadvantages of silica gel storage. It is

limited to sporulating fungi and is unsuitable for *Pythium*, *Phytophthora* and other *Oomycota*, mycelial fungi or fungi with delicate or complex spores. This limitation doesn't apply to yeast cultures. There is a possibility of introducing contaminants by repeated retrievals. Fungi have been stored for in excess of 25 years using this method. Furthermore, all strains assessed to date, have remained genetically stable (Smith *et al.*, 2001).

III.1.5. Soil storage

This technique can be applied to a range of microorganisms that can withstand a degree of desiccation for example the spores and resting stages of filamentous fungi and bacteria such as *Bacillus* spp. The method involves inoculation of double autoclaved soil (121°C for 15 min) with 1ml of spore suspension in sterile distilled water and then incubation at 20-25°C for 5-10 days depending on the growth rate of the fungus. This initial growth period allows the fungus to utilize the available moisture, before the induction of dormancy. The bottles are stored in a refrigerator (4-7°C). Preservation in sterile sandy loam soil may be one of the most practical and cost-efficient ways to preserve filamentous sporulating microorganisms. Other advantages include good viability of cultures for up to 10 years, a reduced chance of mite infection and the option of obtaining repeated inocula from the same source. This method of storage is very successful with *Fusarium* isolates and related genera, although there are reports of some isolates that have been outgrown by mutant strains. Despite this, soil storage should be used in preference to oil storage for the preservation of *Fusarium* species and other fungi that show survival variation when stored under oil. Good recovery has been obtained for *Rhizopus, Alternaria, Aspergillus, Circinella* and *Penicillium* (Smith *et al.*, 2001).

III.1.6. Drying

The cultures conservation method that imitates the natural conditions is drying preservation. It is based on the natural microorganism properties to fall into anabiosis. Sand, soil, mud, active carbon, sawdust, synthetic balls and tablets, polymer matrixes, high disperse materials, filter paper etc. are used as microbic material carrier. The large carrier surface adsorbs part of the moisture. The drying is performed at room temperature or by heating up at 36 – 40 °C. There are references for stability investigations after conservation on different carriers and following drying of representatives of the genera *Shigella, Salmonella, Proteus, Bacillus, Streptococcus, Pseudomonas, Corynebacterium, Rhodococcus, Serratia, Mycobacterium*, with advisable conservation time of 12 months at temperature 4 °C. This preservation method is widely used for brewery and bakery yeasts. A convective drying of starter yeast cultures of species *S. cerevisiae* is applied at 37 °C with ultimate humidity 8 – 10 % (Tsonka and Todor, 2005).

III.1.7. Freeze-drying (Lyophilisation)

Freeze-drying is a highly successful method for preserving bacteria, yeasts and the spores of filamentous fungi. During the freeze-drying process water is removed directly from frozen material by sublimation under vacuum. If carried out correctly, freeze-drying will prevent shrinkage, structural change and help retain viability. There is a vast array of freeze-drying equipment available, ranging from laboratory bench models through to pilot scale and huge industrial installations. Freeze-drying should be optimized for different organisms and cell types. If this is done it should be successful for the majority of bacteria, sporulating fungi, and yeasts. The typical freeze-drying process consists of the following stages:

- material freezing to low temperatures – below the eutectic temperature
- primary drying when the ice crystals sublimate influenced by the passed in the system heat energy in vacuum conditions
- secondary drying when after the ice separation the remained material moisture is desorbed in maximum deep vacuum conditions (Santivarangkna *et al.*, 2011; Tsonka and Todor, 2005).

To monitor freeze-drying a means of measuring vacuum both in the chamber and close to the vacuum pump is required. Comparing the measurements will allow the determination of the end point of the drying process. When the values are equal, water has ceased to evaporate from the material being dried and drying is probably complete. This is confirmed by determining the residual water content. This can be done by dry weight determination or by the use of chemical methods such as the Karl Fischer technique. The freezing point of the material should be determined and the temperature monitored during freeze-drying. The sample temperature must not rise above the melting point until most of the water has been removed (Smith *et al.*, 2001; Tsonka and Todor, 2005).

The technique of centrifugal freeze-drying, which relies on evaporative cooling, can be used successfully for the storage of many sporulating fungi, as well as bacteria and yeasts. However, this is not a method that can be adapted and changed easily, as it is dependent upon the scope of the equipment. Optimization of cooling rate to suit the organism being freeze-dried can be applied using a shelf freeze-drier. There are many advantages of freeze-drying over other methods, including the total sealing of the specimen and protection from infection and infestation. Cultures generally have good viability/stability and can be stored for many years. Ampoules take up little space and can be easily stored. In addition, cultures do not have to be revived before postal distribution. However, there are disadvantages, some isolates fail to survive the process and others have reduced viability and genetic change may occur though unless high viability is retained it is difficult to differentiate between this and selection of spontaneous mutants by freeze-drying. Ampoules of freeze-dried organisms must be stored out of direct sunlight and chilled storage will reduce the rate of deterioration and extend shelflife. However, the process of lyophilisation is relatively complex, can be time-consuming and may be expensive (Smith *et al.*, 2001; Tsonka and Todor, 2005).

From all microorganisms groups the bacteria sustain lyophilisation the best. According to their resistance to drying some authors relate the bacteria to three groups:

- Strongly resistant, such as the genera *Streptococcus, Staphylococcus, Brevibacterium, Corynebacterium, Lactobacillus, Salmonella, Bacillus* etc. Their viability reaches 100 % after drying
- Medium resistant, such as the genera *Brucella, Salmonella, Seratia, Pseudomonas*. Their survival reaches 70 %.
- Sensible to drying are some representatives of the genera *Spirochete, Methylobacter, Methylococcus*

The genera *Aspergillus, Fusarium, Citromyces, Acetobacter, Alcaligenes, Bacillus, Mycobacterium* etc. have a long storage time of 33 – 36 years. The yeasts resistance to freeze-drying and preservation is considerably smaller (Smith *et al.*, 2001; Tsonka and Todor, 2005).

Injury of the cells can occur during the cooling and/or drying stages. The phase changes encountered during the drying process can cause the liquid crystalline structure of the cell membranes to degenerate to the gel phase, which disrupts the fluid-mosaic structure of the membrane. This causes leakage of the membrane, which may culminate in cell damage. Optimal survival can be improved with the use of a suitable suspension medium. Skimmed milk is a suitable protectant for fungi and is sometimes used in combination with inositol. Saccharides such as trehalose protect membranes by attaching to the phospholipids, replacing water and lowering the transition temperature. Other suspending media can be used when preserving bacteria and yeasts with many collections using their preferred preservation base. For example a mix of dextran and trehalose improves the viability of cultures. When the influence of different factors upon bacteria and actinomycetes resistance during lyophilisation is studied the most commonly used protective medium is sucrose 10 % + gelatin. Other media such as 0.1 – 10 % peptone, 10 % sucrose, 10 % lactose, 10 % trehalose, 10 – 20 % skimmed milk, 5 % Na glutamate, casein hydrolysate and many others are applied successfully to the genera *Bacillus, Pantoea, Seratia, Erwinia, Lactobacillus, Acetobacter, Streptococcus* etc. (Smith *et al.*, 2001; Tsonka and Todor, 2005).

III.1.8. Liquid-drying

Liquid-drying is a useful alternative method of vacuum drying for the preservation of bacteria that are particularly sensitive to the initial freezing stage of the normal lyophilisation process. The intrinsic feature of this process is that cultures are prevented from freezing; drying occurs direct from the liquid phase (Malik, 1990, 1992; Smith *et al*., 2001). Also, various selected strains from about 20 species of yeasts, which are reported to be sensitive to freeze-drying and liquid-drying, were successfully dried using a simplified liquid-drying method. All tested cultures proved viable and the majority of the tested strains showed good survival rates after drying. However, different survival levels for different yeasts were observed; generally the sensitivity to drying appeared to be strain-specific. Yeasts that were filamentous, osmotolerant or psychrophilic appeared to be sensitive to liquid-drying and had relatively lower survival levels than the others. Growth and liquid-drying under micro-aerobic conditions resulted in improved survival. The dried yeast cultures proved stable and no mutation or loss in desirable characters was detected. The method can be used for the drying and long-term preservation of nearly all yeast genera (Malik and Hoffmann, 1993).

III.1.9. Cryopreservation

Liquid nitrogen is the preferred cooling agent for cryopreservation, although liquid air or carbon dioxide can be used. Lowering the temperature of biological material reduces the rate of metabolism until, when all internal water is frozen, no further biochemical reactions occur and metabolism is suspended. Although little metabolic activity takes place below -70°C, recrystallization of ice can occur at temperatures above -139°C and this can cause structural damage during storage. Consequently, the storage of microorganisms at the ultra-low temperature of -190 to -196°C in or above liquid nitrogen is the preferred preservation method for most of the culture collections. Provided adequate care is taken during freezing and thawing, the culture will not undergo change, either phenotypically or genotypically. Choice of cryoprotectant is a matter of experience and varies according to the microorganism. Establishing the optimum cooling rate has been the subject of much research. According to some scientists the optimal cooling rate for fungi is 1°C min^{-1}, for yeasts 7-10 °C min^{-1}, for bacteria and actinomycetes 2-45°C min^{-1}. The influence of some physicochemical factors on the yeasts sensitivity to freezing is studied and the slow freezing is recommended – 0.4 °C min^{-1}. In general, cooling at 1°C min^{-1} over the critical phase has proved most successful, but some less sensitive isolates respond well to rapid cooling, preferably without protectant. Slow warming may cause damage owing to the recrystallisation of ice, therefore rapid thawing is recommended. The thawing of different microorganisms is usually in water bath at temperature 25 °C to 41 °C. Slow freezing and rapid thawing generally give high recoveries for fungi (Smith et al., 2001; Tsonka and Todor, 2005).

To reduce the risks of cryo-injury, traditional approaches for cryopreservation have involved controlled cooling at −1°C min^{-1}, typically in the presence of a cryoprotectant such as glycerol, trehalose, or DMSO (Smith and Ryan, 2012). Cryo-injury is a result of several stresses that includes concentration effects caused by pH changes, precipitation of buffers, dissolved gases, electrolyte concentration, intracellular crystallization resulting from loss of the water of hydration from macromolecules, and cell shrinkage. Membrane damage can be a result of concentration effects but may also be caused by ice damage. The physical effects of ice damage can also result in cells becoming ruptured. For microorganisms that are prone to cryo-injury or that exhibit poor viability following preservation, specific protocols can be designed to ensure optimal cryopreservation (Smith and Ryan, 2012).

During cryo-preservation treatment of the cells, the basic damaging factors are the:

- crystallisation of water which causes mechanical disturbances of the cell structures,
- increase in concentration of the cell electrolytes that leads to hydrogen ion concentration changes in the cell and pH disbalance, affecting the cell protein structures, and
- spatial connection between cells and macromolecules that usually do not interact.

Protective compounds – cryoprotectants - are found to eliminate most of the multiple destructive factors during freezing of biological structures. The basic indicator of the protective media is to sustain and/or support the microorganism's viability, morphological, biochemical, taxonomical and genetic properties during conservation and preservation. According to the location of their action, the cryoprotectant media are divided in endocellular and extracellular. The application of protective media with an endocellular mechanism presents a cell penetration. The media overcooling before the freezing contribute to small crystal formation, which restricts the mechanical disturbing action during the cryogenic treatment. The main endocellular protective media are glycerol and dimethylsulphoxid. Some of the extracellular protectants (connecting with the extracellular water) applied to preservation of the biological objects in frozen state are polyvinylpyrrolidone and mostly dextran. The latter can be used combined with other cryoprotectants because it is chemically inert. Combined media for cryo- and lyoconservation and preservation of microorganisms are applied, containing different sugar concentrations (sucrose, glucose, trehalose), colloids (gelatin, agar, peptone, milk and sera), salts (sodium glutamate) etc. In the experiments and in practice, by using combined media, a higher percentage cell viability is established compared to the single component protective media, during and after conservation (Smith *et al.*, 2001; Tsonka and Todor, 2005).

As with other methods of preservation liquid nitrogen cryopreservation has advantages and disadvantages. Advantages include the length of storage, which is considered to be effectively limitless if storage temperature is kept below -139°C. The majority of organisms survive well, giving the method a greater range of successful application. Organisms remain free of contamination when stored in sealed ampoules. Disadvantages of liquid nitrogen storage include the high cost of apparatus such as refrigerators and a continual supply of liquid nitrogen. A regular supply cannot be obtained in some parts of the world and therefore the technique cannot be used. If the supply of nitrogen fails (or the double-jacketed, vacuum-sealed storage vessels corrode and rupture) then the whole collection can be lost. There are also safety considerations to be made, the storage vessels must be kept in a well-ventilated room, as the constant evaporation of the nitrogen gas could displace the air and suffocate workers (Smith et al., 2001). Cryopreservation is the best method for maintaining the genomic integrity of microorganisms; in the future, developments and improvements in preservation methodology should allow the method to be applied for microorganisms that at the current time are difficult to maintain in BRC's (Smith and Ryan, 2012).

III.2. Importance and status of microorganisms culture collections

Culture collections have been around since microbiology began and their basic roles have not changed much over the years (Smith, 2012). They are considered to be a means to preserve microbial diversity *ex situ* (Uruburu, 2003), they are custodians of microbial diversity and play a key role in the storage and supply of authentic reference material for research and development (Arora *et al.*, 2005). They are repositories of strains subject to publication and they carry out safe, confidential, and patent deposit services for researchers. What is changed are the methodologies used to maintain and add value to such resources; this is complicated by the ever-changing legal operational environment through new and revised legislation and the increasing demands of the user (Smith, 2012). The oldest collection that still operates today is the Centraalbureau voor Schimmelcultures, Netherlands; it has recently celebrated 100 years of existence. The American Type Culture Collection (ATCC) is considered the largest supplier of cultures in the world. Collections traditionally hold strains of significant historical interest. There are many European collections. Amongst their holdings are the fungi found on the 5000-year-old Tyrolean Iceman. The most significant accession to the CABI living collection is considered to be IMI 24317, *Penicillium notatum*. Alexander Fleming deposited this as an example of his penicillin producer in April 1945 in the then National Collection of Type Cultures as NCTC 6978 (Arora *et al.*, 2005).

Microbial culture collections are established in many countries around the world. The huge gap between the discovery of new microorganisms and their potential numbers in nature has stimulated an interest in microbial diversity and the harnessing of their genes, properties and products. The operations of microbial collections have changed over the last twenty years as a result of the

advancement of bioinformatics and the facility to present electronic data over the internet. This makes even the smaller collection resources more accessible (Arora *et al.*, 2005).

Culture collection organizations, such as the World Federation for Culture Collections (WFCC), who oversees the World Data Center for Microorganisms (WDCM), and the European Culture Collection Organization (ECCO), act as fora for discussion by bringing together a critical mass of collections and users to try co-ordinate collection activities, enhance their operations as well as to exchange information and technologies that will facilitate progress in this vital task (Smith, 2003). However, it is not just the public service collection that plays a role in ensuring microbial diversity is available, each scientist and each microbiological laboratory has a responsibility here too. The public service collection can support the scientist, but neither may it be possible to maintain the variety of samples the individual needs for their work nor is it possible to have all expertise and facilities available within a single collection. It is absolutely essential that the individual specialist collection preserves these materials well and retains sufficient metadata so that these materials are useful in the future. Culture collections no matter their size, form, or institutional objectives play a role in underpinning microbiology, providing the resources for study, innovation, and discovery (Smith, 2012).

Culture collections vary in size, form and function. They include collections of culturable organisms (e.g. algae, bacteria, fungi, yeasts, protozoa and viruses), their replicable parts (e.g. genomes, plasmids, cDNAs), viable but not yet culturable organisms, cell and tissues, databases containing molecular, physiological and structural information relevant to these collections and allied bioinformatics (Arora *et al.*, 2005). The collections can be small and limited in coverage, collected, and maintained by single researchers; they can be based in laboratories within large multifunctional organizations, and they may be institutional entities developed with the sole purpose of being public service collections that cover a broad range of organisms from many sources. They can focus on organism type, for example, fungi or bacteria and in some instances specific genera; they may have been established to focus on a specific use, for example, industrial enzymes (Polaina and MacCabe, 2007) or antimicrobials or on host crops; they may be linked to a particular sector such as the environment, health care, education, or agriculture. The specific remit, facilities and budget available, and the users they serve define the collection. Some collections are established for a single researcher's use only, for a research group or within an institution, and remain private. Others are open for all scientists to use, and it is these collections that register with the WDCM and make their lists of strains available in hardcopy catalogues or more often, electronically, on the Internet. Many culture collections, that are now described as Biological Resource Centers (BRCs) as defined by the OECD Biological Resource Centre Initiative (OECD, 2001), have the goal to operate according to international quality criteria, carry out essential research, enhance the value and applications of strains, give access to high-quality biological materials, associated information, and services (Smith, 2012).

III .2.1. Culture collection organizations

There are several levels at which co-ordination, collaboration and discussion on approaches to microbial resource collection establishment and organization is carried out. Organizations exist for the support of collection activities on national, regional, and international bases. These include national federations such as the United Kingdom Federation for Culture Collections (UKFCC), United States Federation for Culture Collections (USFCC) and the Japanese Federation for Culture Collections (JFCC). At the regional level the European Culture Collection Organization (ECCO), and at the international level, the World Federation for Culture Collections (WFCC), Microbial Strain Data Network (MSDN), and Microbial Resource Centres (MIRCENs) operate. Collections have also been drawn together to operate at a more intimate level through national and international affiliations such as the Belgium Co-ordinated Collection of Microorganisms (BCCM), the UK National Culture Collection (UKNCC) and Common Access to Biological Resources and Information (CABRI) (Smith *et al.*, 2001).

III.2.2. The World Federation of Culture Collections (WFCC)

The WFCC was founded in 1968 and is a multidisciplinary commission of the International Union of Biological Sciences (IUBS). Since the separation of the International Union of Microbiological Societies (IUMS) from IUBS in 1979 it has operated as an inter-union commission that seeks to promote and foster activities that support the interests of culture collections and their users.

The WFCC has published guidelines agreed by its Executive Board for the establishment and operation of collections. These Guidelines are updated regularly and can be viewed on the WFCC web site (www.wfcc.info/guidelines/). In order that a collection's user can rely upon the organisms supplied, and the services provided, it is imperative that the collection follows good practices. Acceptance of a collection as a member of WFCC offers a limited form of accreditation (Smith *et al.*, 2001).

Member collections of the WFCC register with the World Data Center for Microorganisms (WDCM), and currently there are 627 culture collections registered in 71 countries. A congress is held every 4 years to discuss advances in technology and common policies with regard to biodiversity and the role of culture collections. The WFCC keeps its members informed on matters relevant to collections in its newsletter and has standing committees reporting on patent depositions, postal, quarantine and safety regulations, the safeguarding of endangered collections, education, publicity, standards and biodiversity (Smith, 2003; www.wfcc.info/ccinfo/statistics/).

III.2.3. The World Data Center for Microorganisms (WDCM)

Since 1986, the WFCC has overseen the activities of the WDCM and it is now the data center for the WFCC and Microbial Resource Centers (MIRCENs) Network. The WDCM is supported by UNEP and UNESCO, and the database holds information on collections, the species they hold and details on their specialization. It was established in 1968 and produced the first hard copy volume of the *World Directory of Collections of Cultures of Microorganisms* in 1972, whilst based at the University of Queensland, Australia. The WDCM relocated in 1986 to RIKEN, Saitama, Japan and then again in 1999 to the National Institute of Genetics, Japan. The *World Directory* illustrates some of the data held on the web site (www.wdcm.org); it has indexes by country, main subjects studied, cultures held, the culture availability, their staff, and services offered (Smith *et al.*, 2001).

The WDCM collections hold an excess of 2 million strains (see distribution by continent in Table 5) 47% of which, are bacteria, 27% fungi, 2% viruses, 0.5% cell lines, and 23% others (including plasmids, plants, animal cells and algae) (Smith, 2003). Comparing current data with those of 2003, particularly the number of strains corresponding in each continent, we observe an enormous increase in the numbers of strains in Asia. This is very comforting since several of biodiversity-rich countries are located in Asia such as Malaysia and China (Smith, 2003).

Table 5. World Data Centre for Microorganisms (Smith, 2003; www.wfcc.info/ccinfo/statistics/)

Country	Number of collections		Number of strains		Percent of total number of collections		Percent of total number of strains	
	2003	2012	2003	2012	2003	2012	2003	2012
Africa	10	11	8 540	15 266	2.1	1.8	0.8	0.75
Asia	152	215	215 992	768 647	32.3	34.3	20	37.8
Europe	152	212	470 860	697 599	32.3	33.8	44	34.3
America	111	147	296 496	461 402	23.6	23.4	27	22.7
Oceania	46	42	89 973	89 257	9.7	6.7	8.2	4.4
Totals	**471**	**627**	**1 081 861**	**2 032 171**				

However, the WDCM listings do not include all collections around the world, as there are many private industrial collections and some in independent researchers' laboratories. Of the 627 culture collections, 246 are supported by the governments and 233 by universities; others are considered semi-governmental, private or industrial (www.wfcc.info/ccinfo/statistics/) (Table 6).

Table 6. Categories of culture collections (www.wfcc.info/ccinfo/statistics/)

Supported by	No. of collections
Governmental	246
University	233
Semi-governmental	58
Private	35
Industry	17

The registration of collections with the WDCM facilitates access and traceability of microorganisms and associated data. The registration process requires a unique acronym attached to the collection strains such that, when published in scientific literature, allows the instant recognition of source collection and thus the associated data, for example, the country of origin of the strain. The citation of the WDCM acronym and strain number when the strains are accessed into other collections enables the linkage of information generated on the different lines and avoids duplication of materials in scientific studies. The linkage of strains and the cross-referencing of data are demonstrated by the online tool www.straininfo.net (Smith, 2012). As shown in Table 7, Japan, India and USA hold over 200.000 strains of microorganisms each.

Table 7. Top 20 countries strain holders (www.wfcc.info/ccinfo/statistics/)

Rank	Country	Total hold	Rank	Country	Total hold
1	Japan	232841	**11**	Australia	72040
2	India	216379	**12**	France	69347
3	U.S.A	210276	**13**	Belgium	58252
4	Brazil	145992	**14**	Sweden	52700
5	Korea (Rep. of)	122096	**15**	Germany	51582
6	Netherlands	90775	**16**	Russian Federation	50720
7	China	88373	**17**	Thailand	43106
8	Denmark	86951	**18**	Taiwan	29692
9	U.K.	84132	**19**	Armenia	17805
10	Canada	77841	**20**	New Zealand	16947

III.2.4. Microbial Resource Centres (MIRCENs)

In 1974 UNEP, UNESCO and ICRO established the MIRCEN network and in 1978 the first evaluation of MIRCEN activities was carried out. This led to the establishment of MIRCENs in industrialized countries and the production of MIRCEN News in 1980 to help publicize the activities

of the network. In 1985 Oxford University Press published the MIRCEN Journal of applied microbiology and biotechnology and UNESCO has recommended consolidation and expansion of the MIRCEN network. The objectives of the worldwide network of MIRCENs are to preserve and exploit microbial gene pools, make them accessible to developing countries and to carry out research and development in microbiology and biotechnology (Smith *et al.*, 2001). Currently the UNESCO global network of MIRCENs is comprised of 34 centres.

III.2.5. European Culture Collections' Organisation (ECCO)

The European culture collections have collaborated together since 1982, when the European Culture Collection Curators Organization was established to bring the managers of the major public service collections in Europe together to discuss common policy, exchange technologies and seek collaborative projects. The organization opened itself to staff and users of microorganisms and is now named the European Culture Collections' Organisation (ECCO). There are currently registered 64 collections - 62 corporate and 2 individual - in 24 European countries. The collections currently hold over 350,000 strains. Members have helped produce practical approaches to international rules and regulation. Initiatives led by the Belgian Coordinated Collections of Microorganisms (BCCM) have led to a code of practice for collections to operate within the Budapest Treaty (1983) and the Microorganisms, Sustainable Access and Use, International Code of Conduct (MOSAICC), which provides guidelines for compliance with the CBD (www.belspo.be/bccm/mosaicc). The European Culture Collection's Organisation has incubated initiatives that have helped collections to get support and organise themselves for delivery of products and services. For example, recent European Community Framework Programme projects include the electronic cataloque project CABRI (Common Access to Biological Resources and Information, www.cabri.org), which is setting operational standards for the operation of European biological resource centres (Stern, 2004) and is operating in close collaboration with the OECD BRC initiative and the WFCC, EBRCN (European Biological Resource Centres Network, www.ebrcn.net) and the EMbaRC (European Consortium of Microbial Resources Centres, www.embarc.eu). However, a huge amount of work and investment is still needed by collections, governments and bioindustry, if the power of microbial diversity is to be harnessed effectively (Smith, 2003; Smith, 2012; www.eccosite.org).

III.2.6. Quality Management Systems

There have been several initiatives to design quality management systems for microbial culture collections. The first community-designed system was the WFCC guidelines for the establishment and operation of collections of microorganisms (www.wfcc.info/ guidelines/). National culture collection organizations set up standards, for example, the UK National Culture Collection (UKNCC) quality management system (www.ukncc.co.uk) and various project consortia such as the Common Access to Biological Resources and Information (CABRI) guidelines (www.cabri.org). There are also general standards that can be applied to microbiology laboratories such as Good Laboratory Practice (GLP) and several International Standards Organization (ISO) norms, for example, ISO 17025, ISO Guide 34, and the ISO 9000 series. Although publications on collection management and methodology give information on protocols and procedures, the quality management system must go further and set minimum standards (Smith, 2012). The OECD BRC Task Force considered it essential that a common quality standard was developed for BRCs and that this was to be based upon the existing guidance (OECD, 2007). The OECD best practice covers critical elements in the handling, storage, characterization, and distribution of microorganisms and cell cultures and the handling of associated information. The implementation of best practice introduces controls at all levels of collection operation (Smith, 2012). In order for Microbial Resource Centers (MRCs) to provide reliable high-quality microbial resources and authentic information, the implementation of international criteria in quality management (Arora *et al.*, 2005). Table 8 presents some major MRCs.

While the global culture collectors are debating how to implement these BRC criteria, several collections acknowledge the need to implement recognized standards and have opted for the well-established ISO mechanisms. Comparison with OECD best practice demonstrates that ISO 9001:2000

is not enough to cover BRC operations thoroughly. Although it helps put in place good management systems, it does not address the product or the competence to deliver cultures and associated services. Most often, standards fail to address some of the specific operational requirements of a BRC. Elements often not covered are: (i) compliance with various legal requirements in association with the handling and shipping of biological materials, (ii) the use and preparation of reagents, media, and other supplies, (iii) a strategic plan for BRC future sustainability in order to avoid the loss of biological resources, and (iv) data management and staff qualifications and competence.

Table 8. Some major Microbial Resource Centers (Arora *et al.*, 2005; http://www.wfcc.info/ccinfo/index.php/collection/by_country/).

Country	Institution	Full Name (Acronym)	Status of collection	Homepage
Australia	Women's and Children's Hospital	Mycology Culture Collection (ACH)	Semi-governmental	
Belgium	Laboratoire de Mycologie Systématique et Appliquée, Université catholique de Louvain	Mycotheque de l'Université catholique de Louvain (MUCL)	University	http://www.belspo.be/bccm
Canada	Agriculture and Agri-Food Canada	Canadian Collection of Fungal Cultures (CCFC)	Govermental	http://sis.agr.gc.ca/brd/ccc/
	Labatt Brewing Company Ltd.	Labatt Culture Collection, Technology Development (LCC)	Private	
	University of Alberta	University of Alberta Microfungus Collection and Herbarium (UAMH)	University	http://www.uamh.devonian.ualberta.ca/
China	Institute of Soils and Fertilizers	Agricultural Culture Collections of China (ACCC)	Governmental	http://www.im.ac.cn/institutes/accc/accc.html
	China National Research Institute of Food and Fermentation Industries	China Center for Industrial Culture Collection (CICC)	Governmental	http://www.china-cicc.org
	The University of Hong Kong, Dept. of Ecology and Biodiversity	The University of Hong Kong Culture Collection (HKUCC)	University	
Czech Republic	Institute of Microbiology AS CR	Culture Collection of Basidiomycetes (CCBAS)	Governmental	http://www.biomed.cas.cz/ccbas/fungi.htm
	Department of Botany, Faculty of Science, Charles University, Prague	Culture Collection of Fungi (CCF)	University	
Denmark	Mycology Group, BioCentrum-DTU, Technical University of Denmark	IBT Culture Collection of Fungi (IBT)	University	http://www.biocentrum.dtu.dk/
France	Museum National d'Histoire Naturelle	Fungal Strain Collection, Laboratory of Cryptogamy (LCP)	Governmental	
	University of Bordeaux 2, and INRA	Laboratory of Molecular Genetics and Breeding of Edible Mushrooms (GMACC)	Governmental University	
Germany	DSMZ	Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)	Governmental	http://www.dsmz.de/
Hungary	Corvinus University Budapest	National Collection of Agricultural and Industrial Microorganisms (NCAIM)	Governmental University	http://ncaim.uni-corvinus.hu/
Italy	Dipartimento di Biologia Applicata, University of Perugia	Industrial Yeasts Collection (DBVPG)	University	http://www.agr.unipg.it/dbvpg

Japan	Japanese Federation of Culture Collections	Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University (IFM)	Governmental University	http://www.pf.chiba-u.ac.jp/engli07.html
	Graduate School of Agriculture, Hokkaido University	AHU Culture Collection (AHU)	University	http://www.agr.hokudai.ac.jp/oukin/index.html
		Institute for Fermentation, Osaka (IFO)	Private	http://www.ifo.or.jp/index_e.html
	RIKEN BioResource Center	Japan Collection of Microorganisms (JCM)	Semi-governmental	http://www.jcm.riken.jp/
Netherlands	CBS-KNAW Fungal Biodiversity Centre	Centraalbureau voor Schimmelcultures, Filamentous fungi and Yeast Collection (CBS)	Semi-governmental	http://www.cbs.knaw.nl/
New Zealand	New Zealand Forest Research Institute	Forest Research Culture Collection (NZFS)		http://www.foresthealth.co.nz
	Landcare Research	International Collection of Microorganisms from Plants (ICMP)	Semi-governmental	http://www.landcareresearch.co.nz/databases/db_details.asp?Database_Collection_ID=6
Russian Federation	Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences	All-Russian Collection of Microorganisms (VKM)	Governmental	http://www.vkm.ru/
South Africa	ARC-Plant Protection Research Institute	National Collection of Fungi: Culture Collection (PPRI)	Semi-governmental	
Sweden	Botanical Institute	Fungal Cultures University of Goteborg (FCUG)	University	http://www.systbot.gu.se/database/FCUG/FCUG.html
Taiwan	Food Industry Research and Development Institute	Bioresource Collection and Research Center (BCRC)	Semi-governmental	http://www.bcrc.firdi.org.tw
Thailand	National Center for Genetic Engineering and Biotechnology (BIOTEC)	BIOTEC Culture Collection (BCC)	Governmental	http://www.biotec.or.th/bcc/
UK	CABI Europe UK (Egham)	CABI Genetic Resource Collection (IMI)	Inter-Governmental	http://www.cabi.org/
	PHLS Mycological Reference Laboratory, Central Public Health Laboratories	National Collection of Pathogenic Fungi (NCPF)	Governmental	
USA	University of Missouri, Kansas City, School of Biological Sciences	Fungal Genetics Stock Centre (FGSC)	University	http://www.fgsc.net/
		American Type Culture Collection (ATCC)	Professional Societies, Governmental, Semi-governmental, Private, University Industry	http://www.atcc.org/

Whatever the authoritative document, standard or certification or accreditation process that is selected, a BRC quality management system must address several specific areas such as: organizational requirements; equipment use, calibration, testing, and maintenance records; documentation management; data management, processing and publication; preparation of media and reagents; accession of deposits to the BRC; preservation and maintenance; supply; quality audit and quality review. Among the microorganism domain criteria set down by the OECD are the recommendations on the Best Practice Guidelines on the following: staff qualifications and training; hygiene and biosafety; equipment use, calibration, testing, and maintenance records; preparation of samples; information provided with the biological material supplied.

The overall aim is to implement a program of development that establishes excellence in performance, for culture collections which is the provision of authentic materials, well preserved for future use with valid associated data delivered in a legislative compliant manner (Smith, 2012).

III.2.7. Networking

Networking at the national level is quite commonplace. Most are loose federations bringing together collection staff and users to discuss common issues and share information (Smith, 2012). For example, the UKNCC is linked with nine collections with over 70,000 algae, bacteria, cell lines, fungi, protozoa and viruses (www.ukncc.co.uk). These on-line databases are easy to use and offer a convenient way to look for materials using a simple interface. The UKNCC coordinates some of the activities of the members in marketing and research. Information is provided for each member organization, including inventorying of techniques and equipment. The UKNCC offers both a culture/cell supply service and an identification service (Arora *et al.*, 2005). Others, such as the Belgian Coordinated Collections of Microorganisms (BCCM, www.bccm.belspo.be), are more structured with a governance structure that sets policy and strategy and coordinates operations, research, and development. If a federation of collections is absent in a particular country, microbiologists and collection staff can join regional or global organizations. The WFCC has been promoting culture collection activities for over four decades (www.wfcc.info). Through its web pages, links to the global initiatives and national federations are provided. These are useful information sources that provide assistance not only to the collections themselves but also to facilitate access to users (Smith, 2012).

In addition to providing information, the networking activities have delivered useful output to drive collective development and to underpin research and innovation. The previously mentioned European projects have resulted in technical guidelines and focused information documents covering requirements with which modern day microbial collections are challenged. Importantly, they have provided some of the basic information needed for the OECD BRC initiative that started in 1999. The recommendations of the OECD BRC Task Force toward governments, policy makers, and other stakeholders embraced the importance of safe and legitimate access to high-quality biological materials for research and development. Guidance documents for the operation of BRCs including quality management and biosecurity were developed as well as strategies for setting up a Demonstration Project for a Global Biological Resource Centre Network (GBRCN). This project commenced at the end of 2008 and is supported by the German Federal Ministry of Research and Education (BMBF) following work in the OECD to improve access to high quality biological resources and information to support research and biotechnology as a platform for a knowledge-based bio-economy. The present partnership brings together 15 countries with the World Federation for Culture Collections (WFCC), a global network and regional networks, the European Culture Collections' Organisation (ECCO) and the Asian Consortium for Microorganisms (ACM). On a global level, the project aims at building a structured long-lasting global network, which will pave the way for collections to meet user needs (Smith, 2012; www.gbrcn.org/).

Networking in Europe is moving to the next level with the establishment of MIRRI (Microbial Resource Research Infrastructure, www.mirri.org) which is established on the European Strategy Forum for Research Infrastructures (ESFRI) road map (www.ec.europa.eu/research/infrastructures/index_en.cfm?pg=esfri). MIRRI brings together European microbial resource collections and stakeholders (their users, policy makers, potential funders, and the plethora of microbial research efforts) to improve access to enhanced quality microbial resources in an

appropriate legal framework, thus underpinning and driving life sciences research. ESFRI are establishing these pan-European structures to drive innovation in order to provide the resources, technologies, and services as the basic tools necessary to underpin research. Each ESFRI research infrastructure (RI) is designed to deliver scientific and technological cutting edge and managerial excellence in research, education, and technology and provide clear pan-European added value. RIs are at the center of the knowledge triangle of research, education, and innovation, producing knowledge through research, diffusing it through education, and applying it through innovation (www.mirri.org/esfri.html). MIRRI will integrate services and resources, bridging the gap between the organizations and provision of innovative solutions. It will provide coherence in the application of quality standards, homogeneity in data storage and management, and sharing of workload to help release the hidden potential of microorganisms. MIRRI will enhance existing European microbial collections linking them to non-European country partners globally (Smith, 2012).

III.3. Challenges and opportunities for the conservation of microorganisms in food processing

III.3.1. Enabling and impeding factors in the conservation of microorganisms in food processing

The factors that facilitate and promote the conservation of microorganisms in food processing are closely associated with the protection and further development of culture collections. Those include the exploitation of new technologies, the implementation of international quality standards on culture collections and the networking of the collections.

Relating to new technologies, it is imperative that scientists and researchers in biosystematics and taxonomy employ modern tools of informatics and data processing to make best use of our microbial resources. The elements of taxonomy such as species description, development of identification keys, scientific nomenclature, treatment of morphological, nutritional and physiological traits, are increasingly being computerized to meet the challenge. The process of storage of genetic information with digital techniques for archiving, interpreting and quantifying of data in artificial systems is an important feature of bioinformatics. Sequence data have for many years now been available on the web for public access and utilization (www.ncbi.nih.gov/Genbank/GenbankSearch.html and EMBL (www.ebi.ac.uk/ embl/) are two key resources). Culture collections and MRCs have a role to play in providing information based on authentic and stable strains with validated sequence data. MRCs need to harness the new bioinformatics technologies and begin the networking processes to establish a global network. The groundwork can be laid by stimulating collaborative molecular taxonomic research and novel database development (Arora *et al.*, 2005).

In order for culture collections and those that are becoming MRCs to provide reliable high-quality microbial resources and authentic information, it is essential to implement the international criteria in quality management (Arora *et al.*, 2005). To meet the new demands, culture collections and MRCs have recognized the need to network. National, regional, and international federations have been established and they are formed to further the activities of collections, to facilitate access to their materials and to improve operations. They are most often run on a volunteer basis and rely on individuals finding time to carry out the operations with small amounts of funding, usually from membership fees. Few of the existing organizations have the mandate to change institutional policy and strategy, and this is what is needed if collections are to share tasks and coordinate activities.

In a changing international scientific environment, many collections are under threat of extinction because of inadequate funding, changing government support strategies and the cost of new technologies. We are also suffering a decline in the number of biosystematicians needed to form a sound base for molecular technologies and to aid in identifying, and characterizing microbial diversity (Smith, 2003).

A significant barrier in the conservation of microorganisms in food processing and the related culture collections is the lack of funding. The long-term security of a collection depends on providing a sound financial platform, which is usually a balance between governmental support, commercial, and other income lines. It is a fact that there is a reduction in the availability of governmental funds that is leading to limited core funding. Collections help meet obligations of governments to the CBD

and making available biological resources to underpin science, education, and the economy. Collections protect public funding investments in research by preserving the biological materials generated. Perhaps they should be providing such services under contract. Government funding, when provided, is usually balanced against the income received for the various services and products offered by the collection, with additional income above the plan being returned. This leaves very little resources for investment, to enable the collections, to improve their coverage, and to incorporate new and advancing technologies. Collections need sound and innovative business plans to allow them to keep pace with the ever-increasing demands of science and their users (Smith, 2012).

III.3.2. Ways to address current challenges and suggestions on how to make optimal use of existing opportunities

Going forward there are a few considerations that, if addressed, could strengthen microbial collections. Collections that aim to receive BRC status will need substantial support for establishing and maintaining quality management and accreditation status, as well as for the expansion of research, training and bioinformatics. Any expansion of collection diversity, both in terms of phylogenetic diversity and in-depth coverage at the level of genus and species, will require increased expertise of curators and technical staff. A strategy needs to be outlined which encourages authors to deposit a higher fraction of strains that are currently under-represented in public collections (Stackebrandt, 2010).

Collections need to find novel ways of funding. The traditional business of the general, national, or regional collections must be extended by the provision of new products to meet the needs of today's users. Additional products may include DNA, enzymes, metabolites, and other derivatives from authenticated strains. Collections can move beyond this by developing commercial products through the provision of biotechnological solutions resulting from the discovery of active compounds and funding such activities through public/private investment. The sale of products and services and the delivery of consultancies can be supplemented by research program funding for projects designed to meet donor requirements. However, care needs to be taken in the choice of such activities so as not to divert the collection too far away from their responsibilities in delivering their public services (Smith, 2012).

Collections also need to keep abreast of and harness new technologies to produce information on the strains, adding to their value with the aim of providing today's users with the information they need. It is not always possible to establish these technologies in-house but it is possible to establish partnerships with manufacturers, other collections or institutions with the expertise and the facilities. Bioinformatics is of increasing importance to the operation of collections, and new ways of collecting, storing, analysing and presenting data are required to make best use of biodiversity information. Molecular techniques to differentiate between strains and to aid in their identification are increasing in use. Recent work at CABI has shown through PCR fingerprinting of replicates of an isolate of *Metarhizium anisopliae* that polymorphisms were introduced as a result of non-optimized preservation techniques. Therefore, at the very least collections should be adopting molecular techniques to determine whether they are preserving strains without change (Smith, 2003; 2012). Exploiting existing conservation methods should result to effective and long-term period conservation of microorganisms. The conservation results could be affected by lots of factors during the preliminary culture preparation, by the choice of protectants, preservation and regeneration methods with minimum consequences for the strains (Tsonka and Todor, 2005). In the future developments and improvements in preservation methodology should allow cryopreservation to be applied for microorganisms that are currently difficult to maintain in BRCs (Smith and Ryan, 2012).

It is evident that to better utilize microorganisms facilitating policies, common strategy and sharing workload, resources, and expertise are required. No one single collection can deliver this alone, and therefore, a Research Infrastructure (RI) is being built which will help create the capacity and work more closely with the research community to facilitate access to resources and services with the aim to accelerate the discovery process. There are several issues to be resolved; for example, the majority of the strains cited in the scientific literature should be secured for future use. Project consortia such as EMbaRC and organizations such as MIRRI and the GBRCN are trying to address

these issues and already several Journals are revisiting their policies to try and ensure the biological material on which the published information is based is available for the future. There are policies in place to ensure voucher specimens underpinning microbial taxonomy are preserved and made available for the long term. However, policies to ensure accessibility to those key strains that are required to demonstrate new properties, new findings, and new data and that are reference materials for databases in automated identification systems are still needed. If all the biological materials on which sequences are generated in public databases were available, the anomalies that often occur could be investigated and resolved. Culture collections have a key role to play here.

Additionally, if we are to exploit microorganisms to the full, the much needed data to facilitate this need to be generated and brought together. It is not possible to have all the facilities, expertise, and know how necessary in each individual collection; this needs to be addressed via partnerships. These partnerships should bring together the expertise not only within collections but also throughout the scientific community in general. RIs such as MIRRI, which is pan-European, should be replicated in regions around the world. Examples of activities on which this could be based are seen in Asia, where the Asian BRC Network has been established (www.abrcn.net/); this and the efforts that are ongoing in South America can be brought together with the European RI to build the global network (GBRCN). This would provide a firm footing for microbiology research and development. Using MIRRI as a model, the resulting infrastructures could be governed by the user community with links to research funders to ensure that they deliver in a coordinated and harmonized way, what the research of the day requires. In this way, culture collections and/or BRCs can ensure that isolated and characterized biological materials remain available for future study and use (Smith, 2012).

In this environment, collections must work together to make the best use of new technologies and to contribute to the description of microorganisms yet to be discovered. New technologies and novel ways of funding this task must be engaged and, above all, scientists must collaborate. Common policies are necessary to address the regulatory demands on collections, to control access to dangerous organisms, and, in particular, to enforce the CBD. Countries that hold the majority of biodiversity require support in building the facilities required to explore their hidden resource. The world must benefit from its microbial diversity, which is crucial to solving increasing problems in food provision, public health and poverty alleviation (Smith, 2003).

III.4. Impact of climate change on the conservation of microorganisms in food processing

Various methods and procedures are available and in practical use for the conservation of microorganisms have been mentioned previously. Application of any of these methods, however, requires special technical equipment and an infrastructure to handle, maintain and store microbial cultures. Adequate facilities and skilled staff form an integral part of practically every large-scale (and frequently medium-scale) fermentation enterprise. However, starter culture handling and control may generally not be feasible for small-scale (and particularly artisanal) processors, who, when relying on conventional starters, might have to face extreme hurdles in the case of climate shifts.

Climate change will certainly have no influence on the viability and survival of microorganisms, if preserved and stored under well-controlled technical conditions, and particularly at -80 °C in special storage cabinets.

Even when the present focus of the Svalbard Global Seed Vault is on seed collections, it is hoped that this facility might also serve as a future centre for conservation of technically valuable strains of microorganisms from all over the globe. A major constraint of all culture collections is the availability of space and shortage of funds for energy and staff. All large fermentation industries maintain their own culture collections, also taking care of culture handling and conservation. Examples are given in the footnote (see Section 3.2 for more information) [6].

[6] Nestlé maintains a culture collection of several thousand microbial strains; strong emphasis is placed on R&D. Danone maintains a large collection of beneficial microbial strains, and with strong emphasis on beneficial strains and focused R&D towards health promotion. DuPont/Danisco has a large collection of industrially relevant strains, including commercial cultures for using as starters in the dairy industry, as probiotics, and as protective cultures for bio-preservation of various food products. Christan Hansen maintains a collection of more than 12 000 strains, including commercialised strains for use as probiotics, meat and dairy starters.

In contrast to large enterprises, small-scale processors do not have any means for handling and preparation of axenic starter cultures. In addition, a large proportion of all microbial genetic resources may be contained in traditional small-scale fermentations worldwide. These fermentations are receiving increased attention from researchers worldwide, as is reflected in recent scientific publications, all of which report on the complexity and extreme diversity of the majority of these fermentations (see Tables II to X in Annex 1). On the other hand, only sparse information is available on the microbiology traditional starter cultures (see Table 3), thus strongly justifying increased research focus on their traditional production and on environmental factors influencing microbial community structure and functionality.

Potential starter culture application by small-scale processors has been intensively discussed as an approach for improving the safety and quality of traditional fermented foods. Implementing starter cultures on traditional/artisanal level may become attractive if technical (small-scale and rural) and cost-benefit hurdles can be overcome. Reduction of (energy and other) costs, fermentation time, risk of spoilage (by increased shelf-life), and risk of adverse sensory attributes may contribute to such a development (Holzapfel, 2002).

A potentially complicating factor in delivering standardised starter cultures for traditional food processing may be future climate changes by which the stability and balance within a conventional mixed starter culture can be distorted. The complexity of traditional fermentations has been discussed under Section 2.2, with specific reference to the inter- and intraspecies biodiversity and succession of strains characteristic of a process. Climate-related influences may cause changes in strain balance ratios and a shift in the established species/strain domination in artisanal starter cultures, with a negative effect on the quality and attributes characteristic of the fermented product. The stability and properties of starter cultures traditionally prepared and stored under artisanal conditions will be significantly influenced by changes in environmental conditions, and particularly by temperature and relative humidity.

Traditional back-slopping relies on an inoculum from a previous batch of an active fermentation, containing large numbers of desirable microorganisms adapted to the particular substrate (Holzapfel, 2002). These techniques are the basis of production of yoghurt, kefir, and several other fermented milk products on all continents, and of boza (in Bulgaria) and other fermented cereal based food products, including different traditional home made beers, beer-like fermented drinks, and sourdoughs. Preservation of an inoculum for storage over longer periods may be by dehydration (air- or sundrying) of the fermenting substrate and keeping it in a closed container or plastic bag, frequently after grinding it into a powder. Dehydration will preserve the viability of microorganisms to some extent over relatively long periods, provided the product is maintained in the dehydrated state and not directly exposed to air. On artisanal level, preservation of microorganisms can also be accomplished by involving an inert carrier such as the porous interior of a gourd or calabash, traditionally used throughout Africa, particularly in East Africa, for milk fermentation (see Fig. 11), and other fermentation utensils of natural materials such as wood. An “inoculation belt” is traditionally used in Ghana for initiating pito beer fermentations (Fig. 11).

Typical of several Asian countries are the mixed-culture dough inocula prepared either in the form of dried powders, flat cakes or hard balls (see Fig. 12). They are used for inoculating starchy substrates in the production of alcoholic beverages, and are composed of mixed cultures of filamentous fungi (e.g. *Amylomyces*, *Mucor*, *Rhizopus*, *Actinomucor*), yeasts (*Saccharomyces* spp., *Pichia* spp., *Hansenula* spp.) and LAB (species of *Lactobacillus* and *Pediococcus*) (Tamang, 1998; 2010; Tamang *et al*., 2007), and are referred to by different names in accordance with the region of production. The shelf-life (conservation potential) of these dehydrated starters will depend on:

- the packaging material: e.g., fern leaves, plaintain leaves, paper, synthetic bags,
- the storage (packaging) condition (vacuum, sealed with limited air exchange),
- the temperature,
- other environmental conditions, e.g., humid or dry climate.

It is clear that keeping the quality, and thus the activity, of these artisanal starters will be strongly influenced by climatic conditions. Relying on traditional practices for generations, a changing climate (e.g., increase in temperature and/or changes in relative humidity of the atmosphere) may have a strong impact on the viability and typical population composition of the starter, and thus on the

quality and reliability of the starter.

Figure 11. Examples of the preservation of a traditional starter, involving a porous gourd or calabash as inert carrier (left), used by the Maasai tribe in Kenya for milk fermentation, and an "inoculation belt" (right) traditionally used in Ghana for initiating *pito* beer fermentations (Photographs: Holzapfel).

Figure 12. Traditional preparation (left) and drying (right) of an amylolytic starter known as *marcha* in India and Nepal, used for the inoculation and the alcoholic fermentation of millet to produce "*jaanr*" (Courtesy: Prof. Jyoti Tamang, Gangtok, Sikkim, India).

Traditional starters are prepared in South-East Asia and the Himalayan regions of India, Bhutan and Nepal, by the cultivation of a "natural" consortium of microorganisms, consisting of filamentous fungi, amylolytic and alcohol-producing yeasts and LAB with rice or wheat as base. It is stored in the form of dry, flattened or round balls (Fig. 12) and used for alcoholic production of beverages (Tamang, 2010). These kinds of Asian amylolytic starters are known under different ethnic names such as *marcha* in India and Nepal, *hamei, humao, phab* in India (Tamang, 2010; Tamang *et al*., 1996; Shrestha *et al*., 2002), *mana* and *manapu* of Nepal (Nikkuni *et al*., 1996), *men* in Vietnam (Dung *et al*., 2007), *ragi* in Indonesia (Uchimura *et al*., 1991), *bubod* in the Philippines Hesseltine and Kurtzman, 1990 Hesseltine *et al*., 1988), *chiu/chu* in China and Taiwan (Steinkraus, 1996), *loogpang* in Thailand (Vachanavinich *et al*., 1994), and *nuruk* in Korea (Steinkraus, 1996).

The complexity of the traditional South-East Asian and Himalayan starters is reflected by the microbial diversity in Indian amylolytic starters, comprising filamentous moulds, yeasts and LAB. Representative of the moulds are *Mucor circinelloides*, *Mu. hiemalis*, *Rhizopus chinensis*, and *Rhiz. stolonifer* var. *lyococcus* (Tamang and Sarkar, 1995). The yeasts include *Saccharomycopsis fibuligera, Sm. capsularis, Sacch. cerevisiae, Sacch. bayanus*, *Pichia anomala, P. burtonii*, and *Cand. glabrata* (Tamang and Sarkar, 1995; Tsuyoshi *et al*., 2005; Tamang *et al*., 2007; Jeyaram *et al*., 2008a; 2010), and the LAB are represented by *Ped. pentosaceus, Lb. bifermentans* and *Lb. brevis* (Tamang and Sarkar, 1995; 2007; Hesseltine et al., 1988). The microbial population of *men* of Vietnam includes amylase producers (*Rhiz. oryzae*, *Rhiz. microsporus*, *Absidia corymbifera*, *Amylomyces rouxii*, *Sm. fibuligera*), ethanol producers (*Sacch. cerevisiae*, *Issatchenkia* sp., *P. anomala*, *P. ranongensis*, *Cand. tropicalis*, *Clavispora lusitaniae*), and LAB (*Ped. pentosaceus*, *Lb. plantarum*, *Lb. brevis*, *Weissella confusa*, *W. paramesenteroides*), amylase-producing bacilli (*B. subtilis*, *B. circulans*, *B. amyloliquefaciens*, *B. sporothermodurans*), acetic acid bacteria (*Acetobacter orientalis*, *Ac. pasteurianus*), and some environmental contaminants (Dung *et al*., 2006; 2007; Thanh *et al*., 2008).

Although traditional preservation methodologies are common to many regions and in most cases have a long history, they have not been adequately studied. It would therefore be extremely difficult to reliably predict the impact of climate change on traditional starters. One scenario may be explained by the example presented in the **Annex 2**.

Numerous microbial strains involved in traditional and small-scale food processing operations have been isolated and studied, and the results published either in international SCI Journals and/or compiled in internal reports and documents. However, few of these strains have been deposited in national or well-maintained institutional culture collections, thereby putting at risk the survival, subsequent investigation and future potential for application of these strains. Agar stabs or slant cultures, kept in the refrigerator at ca. 4 °C, are typically used as a first step for strain preservation and maintenance. Yet, most LAB strains have only low survival rates under this set of conditions. Additional obstacles may be energy costs and interruption of electricity, also affecting research institutions and laboratories. Unbureaucratic depositing of these strains in a "Centralised Culture Bank", as discussed under Section II, may offer a partial solution.

Measuring "traits vs. identity", i.e., measuring the community (combined population) features or abilities of the microbes instead of their individual identity, e.g. their resistance to humidity pulses or their ability to function at different temperatures – might be an effective way to study how microbes adapt to climate. Measuring traits may also lead to a better understanding of when a climate-induced adaptation might cause microbes to respond differently to new conditions and affect the way ecosystems function (Sorensen, 2012).

The possible influence of climate change has been studied more thoroughly for soil microbial communities than for microbial populations of food ecosystems. Thus, possible future changes in climate patterns have been modelled for soil microbiota (Evans and Wallenstein, 2012; Wallenstein and Hall, 2012) by monitoring responses to drying and rewetting stress. Bacterial community composition, not differing between rainfall manipulation treatments, became more dissimilar in response to drying–rewetting pulses (Evans and Wallenstein, 2012). By extrapolation, it may be expected that environmental history (e.g., of an artisanal starter) may direct or moderate changes in microbial community structure. Referring to basic constraints on microbial adaptation, specific environments have been suggested where microbial adaptation to climate change (or lack thereof) was considered most likely to alter ecosystem functioning. It is also stated that "the mechanism and level of microbial community adaptation to changing environmental parameters is a function of the potential rate of change in community composition relative to the rate of environmental change". The framework presented by Wallenstein and Hall (2012) for the soil:climate ecosystem presents a valuable basis for developing testable predictions on microbial adaptation (or "functional shifting") to climate within the food regime.

Recommendations:

- Simple methods to preserve and keep traditional starter cultures, include:
  - ✓ packaging in plastic containers/pouches after drying or preparation,
  - ✓ where possible, vacuum packaging,

  - ✓ comparing (as alternative) the suitability of different "inert" carrier materials ("substrates") for immobilising and preserving starters,
  - ✓ further development and standardisation of traditional methods to withstand climatic fluctuations.
- Promotion of small-scale starter culture processing in rural areas by making use of "low-tech." procedures, and supporting local networking among starter providers and small-scale processors.
- Developing models to simulate and measure variation in ratios among key organisms of a traditional starter in relation to temperature, moisture and other climatic factors.
- Training of small-scale producers of traditional starters, e.g. in the basic principles of GHP, GMP, etc., and providing basic equipment for packaging and storage.
- Supporting studies on traditional starter cultures with the objective to:
  - ✓ Characterise the population diversity and strain/species domination,
  - ✓ Identify the strains that are key "role players" in terms of
    - typical quality attributes to the final product, and
    - succession/domination during the different stages of the fermentation process.
- Developing mathematical models for predicting behaviour of the starter (microbial community) as a whole, based on defined "traits" (features) that may serve as indicators of any shift in function or capability. Models for innovative applications in the food regime are presently being developed (McMeekin *et al*., 2008). See also section 2.4.

IV. LOOKING FORWARD: PREPARING FOR THE FUTURE

IV.1. Enhancing the utilisation of microorganisms in food processing

It is clear that many phenotypic differences exist among strains of a certain microbial species. This phenotypic variation among strains has a major impact on their performance in fermentation applications and has been an important source of product diversification and innovation in the past decades (Bron *et al.*, 2011). For example, natural diversity of *L. lactis* provides an important reservoir of industrial functionalities. Genomics and high-throughput technologies provide us for the first time with effective tools for exploiting this diversity for industrial innovations. Comparative analysis of metabolic, regulatory and transport pathways predicted from genome sequences is a valuable tool for predicting strain functionalities. Once template genomes are available, DNA microarrays can be applied for the rapid and cost-effective assessment of gene content in multiple strains. Moreover, by correlating gene content to a variable trait, genetic markers for complex phenotypes can be identified. These approaches yield genetic markers that can subsequently be targeted in miniaturised screening approaches to allow the rapid identification of a strain exhibiting the desired (combination of) properties (Van Hylckama Vlieg *et al.*, 2006).

Recent genomic studies on a wide range of bacteria tell us that the within-species variability can be enormous and it is even speculated that the core genome (core set of genes conserved in most strains of the same species) can be, in some cases, narrower than the pan-genome (auxiliary set of genes variable between strains of the same species). Studies on genomic diversity are, simultaneously, a need and a tool. It is needed to acquire an accurate and better understanding of the community/population dynamics of microorganisms in the digestive track and in traditionally fermented food products and of the role of microbial ecology in food fermentations. Without this knowledge, both the development of probiotics (behaviour and efficacy of allochthonous probiotic strains *versus* autochthonous strains) and our chance to better manipulate or master traditional fermentation processes will be hindered. A comprehensive analysis of the genomic diversity may also serve as an efficient tool to explore the microbial ecology of food products (Zagorec *et al*., 2008).

Novel developments in research lead to effective methodologies and approaches to specifically improve relevant properties of food related microorganisms. Several state-of-the-art biotechnological tools, including the –omics technologies and bioinformatics, along with metabolic engineering and inducible gene expression systems contribute to the above direction. These tools provide attractive possibilities for further optimization of industrial strains. Moreover, recent advances in functional genomics open up the way for assessing side effects of directed mutations and create chances for the development of concrete risk assessment procedures for genetically modified organisms (GMOs) in food. Genomics studies also enable the identification of novel inducible promoters and of new target genes contributing to specific food characteristics, such as flavour and to functional properties related to health (Kuipers *et al.*, 2000).

IV.1.1. "Omics" methods

The current nomenclature of -omics sciences includes genomics for DNA variations, transcriptomics for messenger RNA, proteomics for peptides and proteins, and metabolomics for intermediate products of metabolism. Technological breakthroughs allow simultaneous examination of thousands of genes, transcripts, proteins, and metabolites with high-throughput techniques and analytical tools to extract information. Hypothesis-driven research and discovery-driven research (through -omic methodologies) are complementary and synergistic. Modern screening technologies speed up the discovery process and give broader insight into biochemical events (Blankenburg *et al.*, 2009).

IV.1.1.1. Genomics

The sequencing of the entire genome of numerous microorganisms is driving a revolution in scientific methodology in the life sciences. During the past decade an enormous gain in computing power was achieved, thus paving the way for the holistic, non-targeted study of the keystone biomolecules involved in proper functioning of the cell. No longer does the emphasis lie on the study of individual molecules. Instead, the response of all biomolecules to changes in environmental conditions is being recorded. Subsequently, differences and potential links between the data are identified through bioinformatics (Van der Werf, 2001). Since the early 1990s, DNA sequence production has almost exclusively been carried out with capillary-based, semi-automated implementations of the Sanger biochemistry. Alternative strategies for DNA sequencing can be grouped into several categories. These include **(i)** microelectrophoretic methods such as microchip-based electrophoretic sequencing **(ii)** sequencing by hybridization **(iii)** real-time observation of single molecules, such as nanopore sequencing and the real-time monitoring of DNA polymerase activity and **(iv)** cyclic-array sequencing, such as pyrosequencing (454 platform), direct sequencing of single nucleic acid molecules (Heliscope platform), clonally amplified 1-µm beads that are used to generate a disordered, dense array of sequencing features (SOLiD and the Polonator platforms) and a dense array of clonally amplified sequencing features which is generated directly on a surface by bridge PCR (Solexa technology) (Shendure and Ji, 2008).

The genomics era holds great promise for food biotechnology that involves the bioconversion of raw materials into products that are ultimately consumed. As a consequence of the small genome size of microbes and the availability of today's high-throughput sequencing facilities, microbial genomics is developing fast. However, it is well known that microbial diversity is large, strains adapt quickly, and horizontal gene transfer is a rule rather than an exception. Notably, this also holds true for mobile elements, such as plasmids, bacteriophages and transposons, which are contained in many food-related microorganisms and code for important functions. Furthermore, mixed microbial cultures, and also rotation of single strains (Durmaz and Klaenhammer, 1995; Garneau and Moineu, 2011), are used in many applications, such as several dairy fermentations. Hence, not all microbial production strains can be known at the genome level and there is a need to use comparative genomics in combination with other intelligent and high-throughput genomics approaches (De Vos, 2001).

Mixed-culture food fermentations are of primary economic importance. The performance of such cultures, consisting of LAB, yeasts, and/or filamentous fungi, is not the simple result of "adding up" the individual single-strain functionalities but is largely determined by interactions at the level of

substrates, the exchange of metabolites and growth factors or inhibiting compounds. Technological breakthroughs in the post-genomic era open up new avenues to study microbial communities and interaction networks beyond simple descriptive models. These are now mainly applied to ecological studies of highly complex systems, such as the gastrointestinal tract or complex environmental ecosystems. Food fermentations may provide a valuable alternative model with a high practical relevance. They typically have moderate microbial complexity and offer excellent possibilities for process control. Moreover, the availability of advanced genomics and genetic tools will allow the integration of mechanistic and evolutionary approaches (Sieuwerts *et al.*, 2008).

Genomics generates vast quantities of primary information concerning microorganisms that may be exploited in various ways. In one case, this genomic information may be used to fuel the genetic modification of the host microorganism. The elucidation of metabolic pathways in microorganisms is a first step towards the re-routing of metabolites for natural flavour enhancement in food fermentations. Alternatively, this genomics information may be applied in a more subtle approach that will provide important advantages, without the genetic modification of the target organism, e.g. the understanding of the external factors and responses of *Lb. johnsonii* La1 to stresses suffered during industrial fermentation and processing. In this case, genomics acts as a research tool that is not present in the final product and yet facilitates their improvement. In this respect, genomics has a unique potential as a tool to improve our control of present biological processes and fuel the development of novel products and processes for the food industry (Pridmore *et al.*, 2000).

IV.1.1.2. Metagenomics

Among the methods designed to gain access to the physiology and genetics of uncultured organisms, metagenomics, the genomic analysis of a population of microorganisms, has emerged as a powerful centerpiece. Direct isolation of genomic DNA from an environment circumvents culturing the organisms under study, and cloning of it into a cultured organism captures it for study and preservation. Metagenomic analysis involves isolating DNA from an environmental sample, cloning the DNA into a suitable vector, transforming the clones into a host bacterium, and screening the resulting transformants (Fig. 13). The clones can be screened for phylogenetic markers or "anchors," such as 16S rRNA and *recA*, or for other conserved genes by hybridization or multiplex PCR (136) or for expression of specific traits, such as enzyme activity or antibiotic production or they can be sequenced randomly. A powerful, yet challenging, approach to metagenomic analysis is to identify clones that express a function. Success requires faithful transcription and translation of the gene or genes of interest and secretion of the gene product, if the screen or assay requires it to be extracellular (Handelsman, 2004). New technologies, such as 454 pyrosequencing, have dramatically reduced sequencing costs, to a level where metagenomic analysis may become a viable alternative to more-focused assessments of the phylogenetic (e.g., 16S rRNA genes) and functional diversity of microbial communities (Wommack *et al.*, 2008).

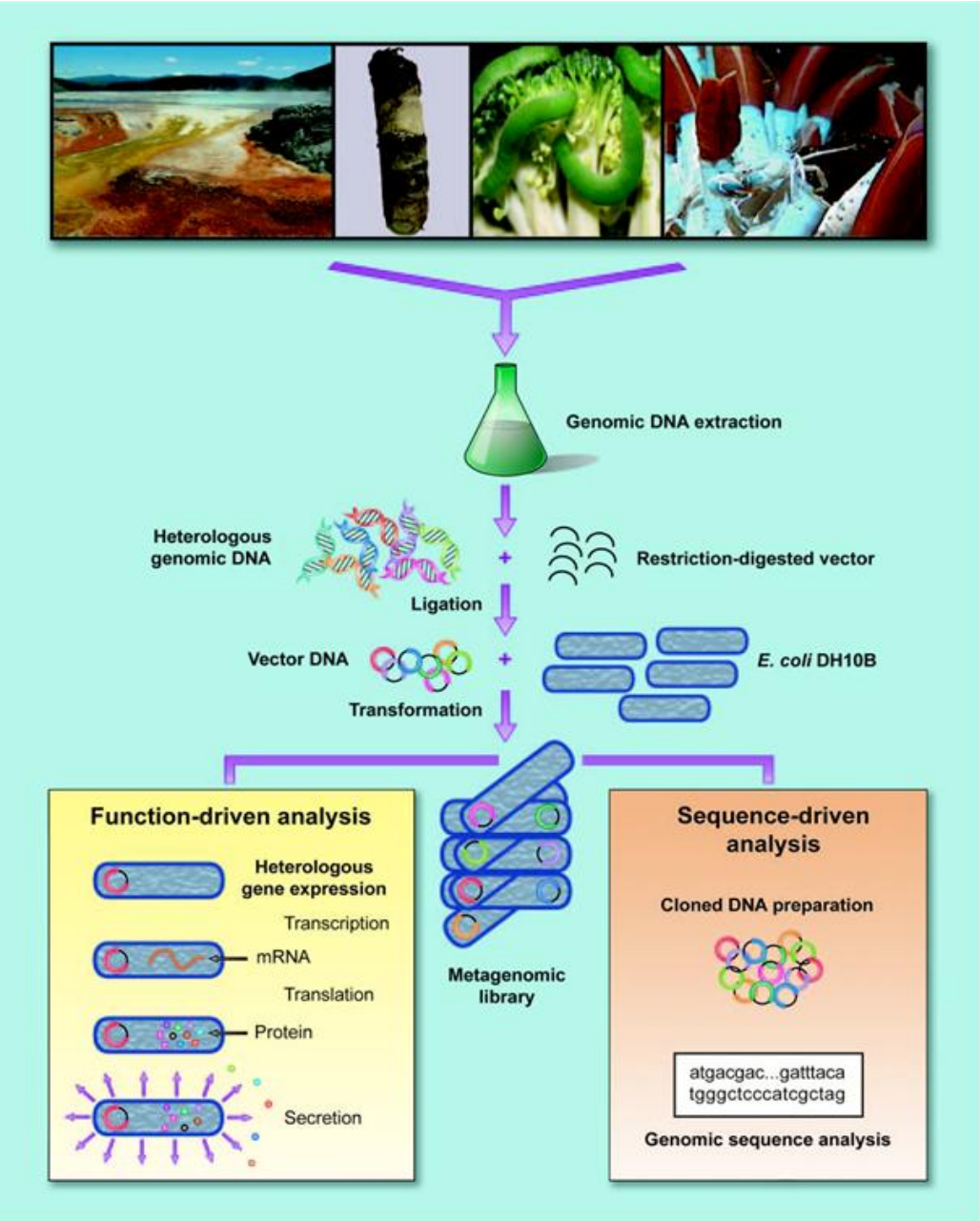

Figure 13. Construction and screening of metagenomic libraries. Schematic representation of construction of libraries from environmental samples. The images at the top from left to right show bacterial mats at Yellowstone, soil from a boreal forest in Alaska, cabbage white butterfly larvae, and a tube worm (Source: Handelsman, 2004).

In a recent study, metagenomic approaches were used to monitor changes in bacterial populations, metabolic potential, and overall genetic features of the microbial community during the 29-day fermentation process in Kimchi, a traditional food in the Korean culture, made from vegetables by fermentation. Phylogenetic analysis from the metagenome indicated that the kimchi microbiome was dominated by members of three genera: *Leuconostoc*, *Lactobacillus* and *Weissella*. Besides microbial genome sequences, a surprisingly large number of phage DNA sequences were identified from the cellular fractions, possibly indicating that a high proportion of cells were infected by bacteriophages during fermentation. Overall, these results provide insights into the kimchi microbial community and also shed light on fermentation processes carried out broadly by complex microbial communities (Jung *et al.*, 2011). This kind of research can be applied to other types of fermented foods.

Food industry motivations to probe the enormous resource that is uncultivated microbial diversity, the dimensions of which are truly astonishing, rely on a global political drive to promote white (industrial) biotechnology as a central feature of the sustainable economic future of modern industrialized societies. This requires the development of novel enzymes, processes, products and applications. Metagenomics promises to provide new molecules with diverse functions. Together with *in vitro* evolution and high-throughput screening technologies, metagenomics provide industry with an unprecedented chance to bring biomolecules into industrial application (Lorenz and Eck, 2005).

IV 1.1.3. Functional Genomics

The recent "explosion" in genome sequencing of food-related microorganisms (Mayo *et al.*, 2008) has opened the way for functional genomics approaches, including transcriptome, proteome and metabolome analyses, as well as structural genomics. Figure 14 gives an overview of approaches involved in functional genomics programs and their expected deliverables for food biotechnology. New improvements in large-scale sequencing methodologies are continuously being reported, indicating that even faster accumulation of sequencing data is to be expected. In order to efficiently exploit this information, novel high throughput analytical methods are being developed, such as DNA microarrays (transcriptome) and improved 2D-electrophoresis methods combined with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopic analysis (proteome). The huge number of experimental data generated is gathered into large databases, the interpretation of which greatly depends on novel bioinformatics methodologies, which should enable linking of the databases and facilitate classification and interpretation of the results (Kuipers, 1999).

Stimulated by the rapidly increasing number of completed genome sequences of food-related bacteria (e.g. starters, probiotic strains), and those expected to be finished soon, functional genomics approaches are being developed for employing these data to the benefit of the food industry. Functional genomics approaches are extremely well suited to gaining a better understanding of cell functioning and physiology in a product or host environment. Even when only a 'gapped' genome sequence is available (e.g. 3.2 times coverage by random sequencing), specific methods for analysis can still give a good overview of the central metabolism, similar to that obtained from a complete genome sequence. Also, computational methods to make optimal use of available sequences, combined with expression data, are being developed that can predict functional interactions between proteins and can help to assign previously unknown functions to proteins. In view of the relatively small genome sizes of bacteria and their good amenability to genetic manipulations they make excellent model systems for transcriptome and proteome analysis, and for comparative genomics (Makarova *et al.*, 2006), addressing important questions on, e.g., side effects of genetic modifications, mechanisms of antimicrobial resistance development, industrial stress responses, improved cell factories, metabolic pathway engineering, probiotic functions and host-microbe interactions. Bioinformatics tools to study the properties of metabolic networks are being developed, for comparative functional analysis of microbial genomes, which will be extremely useful for comparing traits of industrial microorganisms (Kuipers *et al.*, 2000).

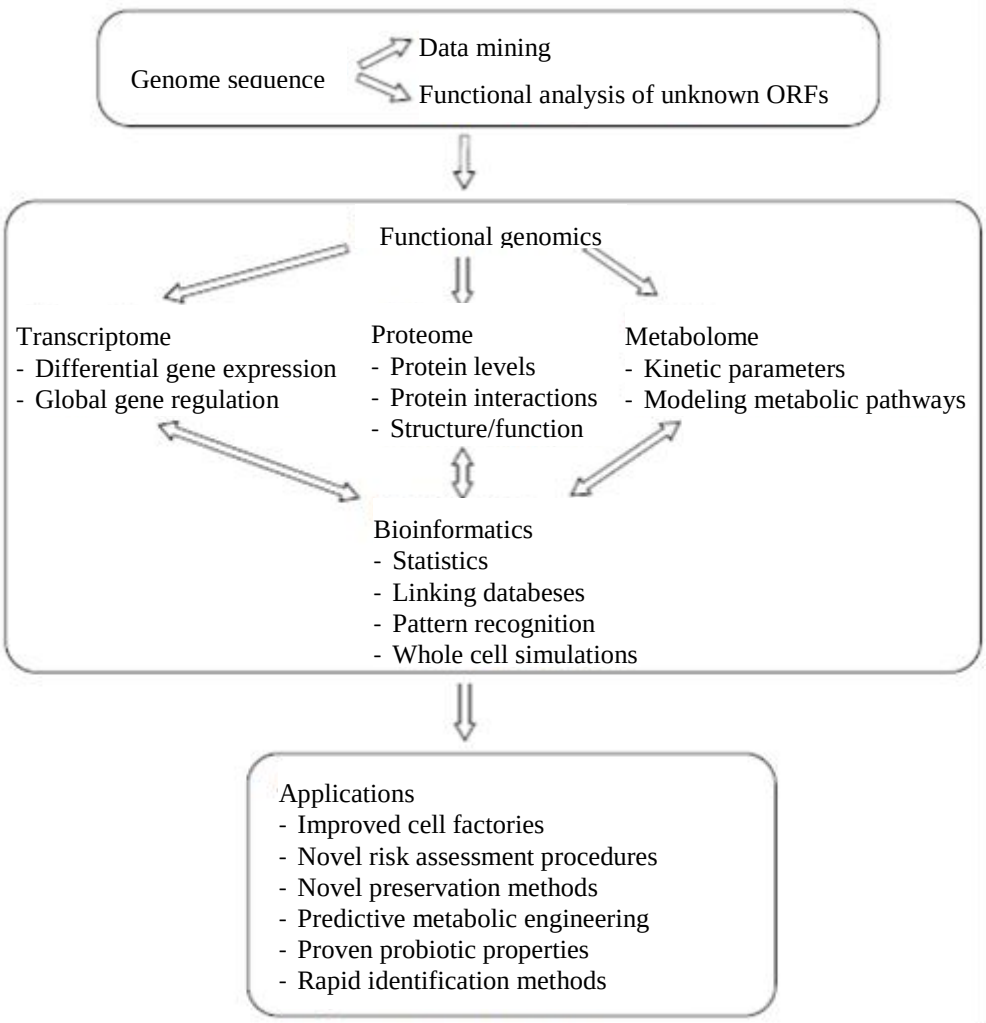

Figure 14. Functional genomics of food microorganisms (Source: Kuipers, 1999).

IV.1.1.4. Transcriptomics

The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease. The key aims of transcriptomics are: to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs, to determine the transcriptional structure of genes, in terms of their start sites, 5' and 3' ends, splicing patterns and other post-transcriptional modifications and to quantify the changing expression levels of each transcript during development and under different conditions (Wang *et al.*, 2009).

Several high-throughput RNA measurement tools such as differential display, transcript imaging, and DNA microarrays have been developed for the analysis of the transcriptome (Han and Wang, 2008). The use of next-generation sequencing technology to study the transcriptome at the nucleotide level is known as RNA-Seq (Wang *et al.*, 2009). DNA microarrays have been successfully used to examine whole-genome expression profiles of bacteria grown under various environmental conditions, such as exposure to alternative carbon sources, stress and starvation conditions, exposure to solvents, varied pH-values, and during the production of metabolites and recombinant proteins (Park *et al.*, 2005). All this information may find application in the improvement of microorganisms used in industrial and, more importantly, in traditional food processing. These data could be used to build quantitative food ecosystem models that link microbial community dynamics with the biochemical functioning of the community and thereby predict changes in microbial processes

associated with climate changes, an approach proposed by Schimel and Culledge (1998) for soil microbial communities.

The application of transcriptomic methods to microbial communities is creating a new research agenda in which sequence data are the analytes in experimental field studies. This approach allows the measurement of gene expression in microbial assemblages, in microcosms, mesocosms or natural samples, as a function of environmental variability over time. By tracking the genes responsive to specific environmental perturbations, it should soon be possible to track environmental variations that are first observed as changes in gene expression, but may lead later to shifts in community composition. Quantifying the variability and kinetics of gene expression in natural assemblages has the potential to provide a fresh perspective on microbial community dynamics (DeLong, 2009). With the advent of various high-throughput techniques that aim to characterize complete microbial ecosystems (metagenomics, meta-transcriptomics and meta-metabolomics), it is time to consider molecular systems biology at the ecosystem level (eco-systems biology) (Raes and Bork, 2008).

IV.1.1.5. Proteomics

Proteomics is considered to be powerful enough to help to find a function for proteins annotated in a fully sequenced genome. The classical approach is to delete the gene encoding the protein of interest and to observe phenotypic variation between wild type and mutant strain. Proteomics gives a new dimension to this kind of study by allowing one to observe and quantify the amount of synthesis of a great number of cell proteins (Champomier-Vergès *et al.*, 2002).

The use of proteomic tools allows a global and dynamic view of proteins that are expressed by bacteria. As an increasing number of bacterial genomes are currently available for homology searches, it is now possible to use such techniques to screen proteins expressed by microorganisms used in various fermented foods. Proteomic tools are also useful to investigate protein heterogeneity in protein-rich foods. Proteomics is a powerful tool for analysing several hundreds of proteins simultaneously in complex mixtures. High performance liquid chromatography (HPLC) is another technique of choice for proteomic studies, especially for protein identification through peptide analysis, due to its ability to separate and identify lower molecular mass molecules. Multidimensional HPLC and tandem MS coupled on-line are systems that are capable of separating and identifying low-abundance and membrane proteins which escape 2DE analysis (Manso *et al.*, 2005).

Proteome of starter cultures in fermentation processes (beer, cheese, sausage, etc.) can be also used to predict the quality of the fermented end-product (Han and Wang, 2008). Proteomic tools have been used for the characterization of milk proteins and in the study of protein expression of lactic acid bacteria used for manufacture of dairy. Also they have been applied to cheese as an example of a complex food matrix [a mixture of animal (milk) and microbial proteins], focusing on a novel strategy that allows the study of the enzymatic machinery found in situ in cheese (Manso *et al.*, 2005). For example a proteomic view was obtained for different groups of proteins within the Emmental cheese using proteomic tools to create a reference map. The five functional groups of proteins that were identified are involved in: (i) proteolysis, (ii) glycolysis, (iii) stress response, (iv) DNA and RNA repair and (v) oxidoreduction. The results revealed stress responses triggered by thermophilic lactic acid bacteria and *Propionibacterium* strains at the end of ripening. Information was also obtained regarding the origin and nature of the peptidases released into the cheese, thus providing a greater understanding of casein degradation mechanisms during ripening. Different peptidases arose from *Strep. thermophilus* and *Lb. helveticus*, suggesting that streptococci are involved in peptide degradation in addition to the proteolytic activity of lactobacilli (Gagnaire *et al.*, 2004).

IV.1.1.6. Metabolomics

Metabolomics is an emerging field of -omics research that focuses on high-throughput characterization of small molecule metabolites in biological matrices. There are many approaches for metabolomics. They can be roughly classified according to a data quality and a number of metabolites that could be detected. First is the "**metabolite targeted analysis**" that refers to the detection and precise quantification of a single or small set of target compounds. Second is the "**metabolic profiling or metabolite profiling**", which provides the identification and approximate quantification of a group

of metabolites associated to specific pathways. Metabolic profiling aims at the quantification of preselected metabolic pathways or groups of metabolites with similar chemical properties and promises to be an effective method for investigating microbial metabolism in a quantitative manner. Third is "**metabolite fingerprinting**". It is used for complete metabolome comparison without knowledge of compound identification. Usually, a spectral analysis is used in fingerprint analysis (Krastanov, 2010).

Unlike gene-expression studies or proteomics analyses, which only reveal part of what might be happening in a cell, metabolomic profiling can give an instantaneous snapshot of the entire physiology of that cell. More importantly, if data from proteomics, transcriptomics and metabolomics can be integrated, a more complete picture of a living organism's biology can be obtained. Metabolomics also represent a useful tool for functional genomics programmes. In order to elucidate an unknown gene function, genetic alterations are introduced in a system. By analysing the phenotypic effect of such a mutation (i.e. by analysing the metabolome), functions may be assigned to the respective gene. Sampling would be performed once for every genotypic mutation and the analysis of the metabolome would optimally include an identification and quantification of all metabolites of a biological system (Krastanov, 2010). The metabolome measurement efforts were largely put on the model organisms such as *Sach. cerevisiae*, *E. coli*, *Bacillus subtilis*, etc. (Wang *et al.*, 2006).

The enhanced resolution provided by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), along with powerful chemometric software, allows the simultaneous determination and comparison of thousands of chemical entities, which has led to an expansion of small molecule biochemistry studies in microorganisms. Continued development of these analytical platforms will accelerate the widespread use of metabolomics and allow further integration of small molecules into systems biology (Idle and Gonzalez, 2007). Metabolic control analysis (MCA) and metabolic flux analysis (MFA) are the key components of metabolic engineering. Quantified intracellular metabolites can be used to address the in vivo reaction kinetics of one reaction, the reactions in one pathway, or the global regulatory metabolic control mechanisms directly. The basic approach used here is to quantify exactly the metabolite difference in microorganism under different phenotypes (usually genetic or other perturbations). Comprehensive profiling of all the metabolites with very rapid sampling frequency is indispensable for such purpose. As illustrated before, mathematic models and standard data set can also greatly facilitate this process. Undoubtedly, metabolomics will have a strong impact on microbiology in the coming decades. However, the power of metabolomics will largely rely on how to improve the comprehensible metabolite coverage, construct a reference material database, and facilitate integration with different -omics data sets. Obviously, it is far from our target. But at least, metabolomics has shed light on one new approach for future exploring of the metabolic complexity of microorganisms (Wang *et al.*, 2006).

IV.1.2. Bioinformatics

Bioinformatics can be defined as the use of computers for the acquisition, management, and analysis of biological information. Bioinformatics combines *in silico* biological techniques with the DNA sequencing analysis approach. *In silico* biology combines statistical and mathematical algorithms with the need to manage and elaborate huge numbers of biological data (Giraffa and Carminati, 2012). The huge number of experimental data generated by the use of –omics is gathered into large databases, the interpretation of which greatly depends on novel bioinformatics methodologies, which should enable linking of the databases and facilitate classification and interpretation of the results. Fast developments are also taking place in bioinformatics. Systems for cluster analysis and display of genome-wide expression patterns have been developed that provide information on the status of cellular processes and on the possible functions of genes without a known functionality. Novel data-mining and -linking approaches get increasing attention, yielding methods for determining microbial genescapes. In addition, bioinformatics could allow advances in functional genomics, e.g. conversion of the mass of sequence data presently available in public databanks into knowledge, so that microbial diversity could be assessed not only at the molecular level, but also at the functional level (Kuipers, 1999).

Computational modeling of biological systems is becoming increasingly important in efforts to better understand complex biological behaviours. An exciting development in bioinformatics is the building of an integrative model called E-CELL, for simulating intracellular molecular processes to predict the dynamic behavior of cells, incorporating information on gene regulation, signalling and metabolism. It is equipped with graphical interfaces that allow observation and visualization of interactions during cellular processes. The system gives the user the opportunity to define functions of proteins, macromolecular interactions, and characteristics of cellular metabolism as a set of reaction rules and eventually allows for *in silico* experimenting. The system can continuously be improved by adding novel experimental data, which are currently generated at a high pace. Another interesting project is called 'the Virtual Cell', which aims at modelling of cellular processes to be used as a tool for experimental biologists, for example, for metabolic engineering purposes. The input comes from users providing biochemical and electro-physiological data on any cell of interest. The powerful approach in functional genomics of using knowledge coming from metabolic engineering studies and gene–phenotype relationships for model building forms a major challenge in bioinformatics (Kuipers, 1999). The Self-Organising Map (SOM) is an effective tool for clustering and visualizing high dimensional complex data on a single map. SOM recognized species-specific characteristics (key combinations of oligonucleotide frequencies) in sequence fragments permitting species-specific classification (self-organization) of the sequences without any information regarding the species. Because species-specific clustering on SOMs is very clear, SOMs are a powerful tool for phylotype classification of genomic sequences, especially sequence fragments obtained from mixed genomes of uncultured environmental microorganisms (Abe *et al.*, 2006).

Further developments in bioinformatics are ongoing, especially in the fields of genome mining, including functional analysis of genes with known and unknown function, linking databases with experimental data (transcriptome, proteome, metabolome, protein structure and function) and pattern recognition (Kuipers, 1999).

IV.1.3. Metabolic engineering

Metabolic engineering has been defined as the direct improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology. Therefore the analysis and modification of metabolic pathways is of central importance in ME. Because of the significant role of fermentation in food production and preparation, microorganisms are constantly encountered in these processes. ME can rationally improve the properties of these microorganisms and their efficiency for producing different products (Gonzalez, 2005).

Metabolic engineering is enjoying a high profile thanks to a number of recent developments (Figure 15).

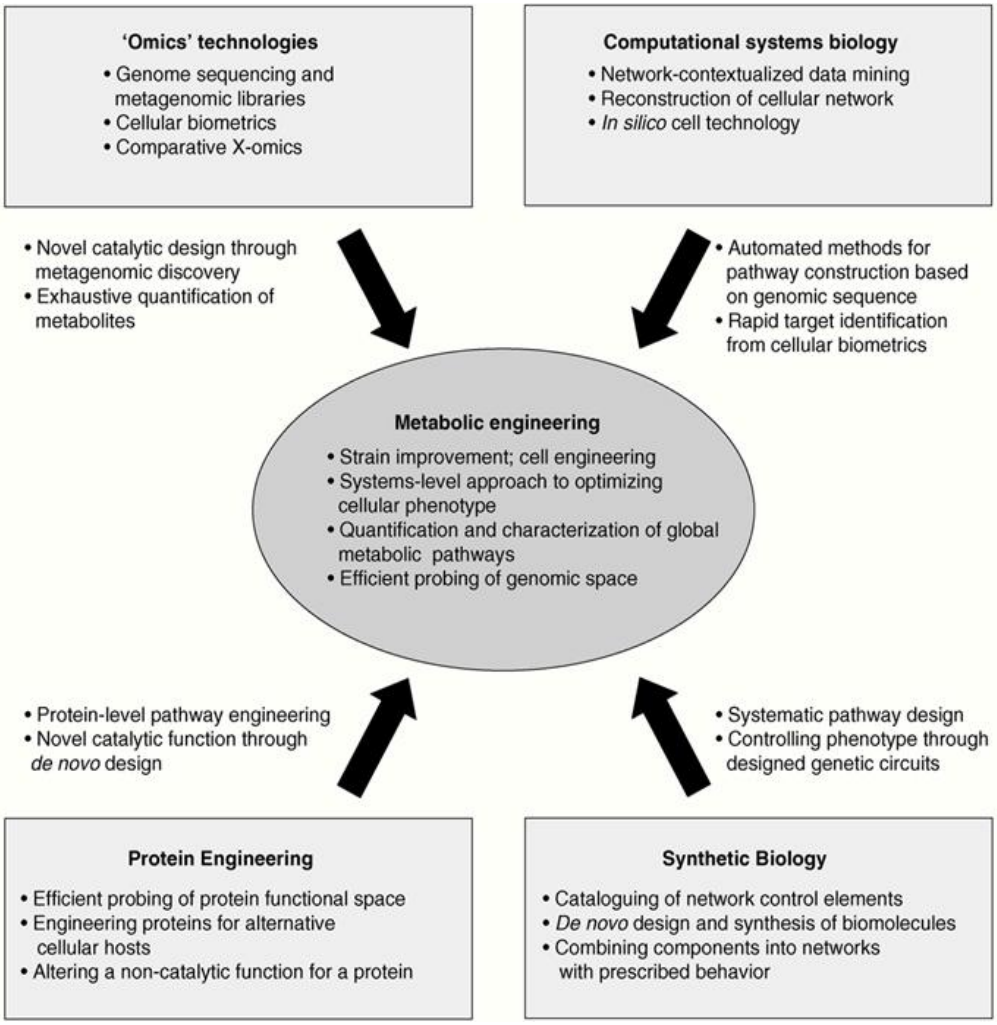

Figure 15. Metabolic engineering gains from advances in complementary biological fields (source: Tyo *et al.*, 2007).

Omics technologies and computational systems biology can provide large amounts of data about a cellular state and the means by which to analyse it, whereas protein engineering and synthetic biology can provide toolsets for new ways to manipulate a cell to improve the cellular properties (Tyo *et al.*, 2007). The advances in these fields became obvisouy due to the rapid elucidation of the complete nucleotide sequences of (microbial) genomes and the possibility of examining gene expression patterns on a whole genome scale by DNA microarrays, and by serial analysis of gene expression (SAGE). One of the real challenges is to devise a bioinformatics framework for data management and analysis aimed at extracting as much information from the overwhelming quantity of raw data as possible. Stoichiometric models, such as Metabolic Flux Analysis (MFA), and kinetics models aimed at describing overall cellular functions or individual processes, complement statistical and cluster analyses (Gombert and Nielsen, 2000; Kuipers *et al.*, 2000).

Information from fully sequenced genomes makes it possible to reconstruct strain-specific global metabolic networks for structural and functional studies. In order to properly understand and analyse the global properties of metabolic networks, methods for rationally representing and quantitatively analysing their structure are needed. The elementary flux mode is a mathematical tool that can be used to define and describe all metabolic routes that are stoichiometrically and thermodynamically feasible for a group of enzymes. It can be used to optimize yields in biotechnological applications, to identify functions for orphan genes and to convert a list of putative enzymes derived from the annotation of a complete genome sequence into a set of metabolic pathways. Automated computational analysis techniques are being developed for predicting the metabolic pathways of a microorganism from its genome sequence and for producing integrated

pathway-genome databases that model the resulting predictions (Kuipers *et al.*, 2000; Ma and Zeng, 2003).

Applying ME principles and tools to the production of food ingredients by microorganisms has resulted in the efficient production of both native and totally novel products by several cultures including strains of LAB, *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium glutamicum* (Table 9). Some examples of genetically engineered microorganisms and their applications in food prosesses are given in Annex 2.

Table 9. Examples of bacteria that have been engineered for the production of food ingredients (Gonzalez, 2005).

Microorganism	Product
Corynebacterium glutamicum	L-tryptophan, L-phenylalanine, L-tyrosine, L-threonine, L-isoleucine, L-histidine
LAB	L-alanine, Vitamins, Exopolysaccharides, Bacteriocins, Aroma Compunds
Bacillus subtilis	Vitamins, Lactic acid
Escherichia coli	Succinate, Pyruvate, Lactate, Acetate

The discipline of metabolic engineering has been subject to continuous enhancements. There have been dynamic developments of available tools for modifying, analysing, and modelling metabolic fluxes. It is also increasingly obvious that improving cellular activities requires a holistic view of the entire cell rather than isolated metabolic pathways. In addition, other engineering disciplines such as inverse metabolic engineering or evolutionary engineering have facilitated rational metabolic engineering (Table 10) (Nevoigt, 2008).

Table 10. Engineering approaches for microbial strain improvement (Nevoigt, 2008).

Strategy	Description	Advantage(s)
Rational metabolic engineering	Engineering metabolic pathways (mainly enzymes, transporters or regulatory proteins) based on available information	Known genetic modifications leading to strain improvement can be transferred to other strains
Inverse metabolic engineering	Starts from the phenotype; analysis of the genetic basis for the phenotype; transfer of the genetic basis to the production strain	Independent of preliminary knowledge about pathways, enzymes, and their kinetics; can reveal novel, unknown target genes for improvement
Random engineering strategies (support inverse metabolic engineering)		
Evolutionary engineering	Includes all random genetic modifications and perturbations; relies primarily on random mutagenesis of the entire genome or its parts; identification of clones with improved phenotypes via appropriate screening methods;	independent of preliminary knowledge about pathways, enzymes, and their kinetics; multigenic traits can be addressed

	identification of genetic modifications is difficult (except when a limited DNA sequence is mutated)	
Transposon mutagenesis and gene overexpression libraries[a]	Random deletion or overexpression of single genes; identification of clones with improved phenotypes by appropriate screening methods	independent of preliminary knowledge about pathways, enzymes, and their kinetics can reveal novel, unknown target genes for improvement; genetic modifications can be easily identified
Global transcription machinery engineering	Mutagenesis of basal transcription factors leading to a global reprogramming of gene transcription; identification of clones with improved phenotype by appropriate screening methods	Independent of preliminary knowledge about pathways, enzymes, and their kinetics multigenic traits can be addressed the genetic modification that triggers the optimal global reprogramming can be identified and transferred to another strain

[a] These strategies can, in principal, also be defined as evolutionary engineering, but they represent special cases

IV.1.4. Stress induced gene expression

The ability of starter microorganisms to respond to and withstand technological stresses is essential for an incessant fermentation process and can seriously influence the overall quality of the final product.

Stress can be defined as a change in the genome, the proteome or the environment that results in a decrease in the growth rate or survival of a microorganism. Stress responses are extremely important for microorganisms, which experience continual changes in factors, such as temperature, nutrient and water availability or osmotic pressure in the environments in which they occur. The physiological status of the cells and environmental factors will affect the mechanism of response to stress. The activation of defences against stress conditions depends on regulated gene expression. Regulators often control several genes and sometimes even other regulators. Bacterial stress responses therefore rely on the coordinated expression of genes that alter cellular processes such as cell division, DNA metabolism, housekeeping, membrane composition metabolism and transport, acting in concert to increase the bacterial stress tolerance (Franz and Holzapfel, 2011).

Genes implicated in stress responses are numerous and in food related microorganisms the levels of characterisation of their actual role and regulation differ widely between species. A better understanding of the mechanisms of stress resistance should allow to understand the bases of the adaptive responses and cross protection, and to rationalize their exploitation to prepare microorganisms to industrial processes. Moreover, according to van de Guchte *et al.* (2002), the identification of crucial stress related genes will reveal targets for:

- specific manipulation (to promote or limit growth)
- developing tools to screen for tolerant or sensitive strains and
- evaluating the fitness and level of adaptation of a culture.

This identification could be further exploited for monitoring the evolutionary adaptive changes in microorganisms in response to climatic stresses, since stress related genes can be used as marker genes. With this approach, the following could be identified:

- weak strains with low expression of stress related genes, which are threatened with extinction under climatic changes. For the conservation of those strains a databank could be created in order to preserve microbial diversity, and
- strong strains with high expression of stress related genes and therefore with greater chances of survival under changing climatic conditions.

Although the occurrence of various stress inducible systems is clear, further characterization of the links between the regulatory networks is necessary to be able to fully exploit them. The use of proteomics and DNA arrays will provide a more accurate global view of gene regulation under different environmental or industrial stresses. These studies will likely result in the development of new expression systems activated under industrial conditions and will allow the construction of bacterial strains resistant to multiple stresses, while producing the desired metabolites (Kuipers *et al.*, 2000).

IV.2. Potential new roles and uses of microorganisms in food processing and possible future research

Innovation in the starter culture industry is stimulated by possibilities and needs. New possibilities are constantly being opened up by rapid developments in the biological sciences. Our ability to understand complex biological systems has been transformed through the invention of methods to accumulate and analyse large amounts of data. The genomes of several microorganisms have now been completely sequenced, including several pathogenic bacteria as well as some of the microorganisms used in food fermentations (e.g. *Saccharomyces cerevisiae* and *Lactococcus lactis*). Safe methods for the genetic engineering of food microorganisms have been developed for the most important species, and this has opened a wide range of possibilities for the improvement of yeast and lactic acid bacteria metabolism. The practical applications of the modern methods in Europe however, have been delayed due to public resistance to modern biotechnology. The other factor stimulating innovation is the need for new products. There is a big need for new methods to preserve crops after harvest and reduce spoilage before consumption. This need is very strong in the less developed world, but also in the highly developed countries better methods are needed to extend shelf life and avoid spoilage. Food fermentation and bioprotective cultures can solve some of these problems.

Probiotic cultures with specific health benefits with defined modes of action, is also an area where the market would welcome new products. These two examples are specific areas under the more general need for new cultures or new cultures formulations in order to expand the use of beneficial microorganisms in food (Hansen, 2004).

IV.2.1. Probiotics and functional foods

Probiotics are becoming more and more accepted in clinical practice, where they are successfully employed in the management of a variety of diarrheal disorders, including rotavirus diarrhoea, antibiotic-associated diarrhea, *Clostridium difficile* diarrhoea (McFarland, 2006) and traveller's diarrhoea (McFarland, 2007). New data suggest that probiotics might be useful as adjuvants in controlling inflammatory diseases (Ewaschuk and Dieleman, 2006) treating and preventing allergic diseases (Kalliomäki *et al.*, 2010), preventing cancer (Kumar *et al.*, 2010), and stimulating the immune system, which may reduce the incidence of respiratory disease. Such effects could justify the addition of not one but potentially several probiotics to commonly consumed foods, which could achieve population-wide health benefits. Genomic and proteomic approaches will help to identify key molecules and to define novel targets of probiotic action (Schiffrin and Blum, 2001; Vanderhoof, 2001). Different modes of administering probiotics are currently being investigated, which may ultimately lead to the widespread use of probiotics in functional foods. It is important that such practices be directed by carefully controlled clinical studies published in peer-reviewed journals (Vanderhoof, 2001).

Controlled human studies are essential for the success of probiotic functional foods, and they should be tailored for specific population groups such as the elderly and babies (Coman *et al.*, 2012).

Future research on probiotic bacteria will centre on selecting new and more specific strains for the well-being of the host (age groups, healthy populations, disease specific). The future scientific and technological research trends will be (Mattila-Sandholm, 2002):

- to study the mechanisms of action of probiotics in the GI-tract, and develop diagnostic tools and biomarkers for their assessment.
- to examine the effects of probiotics on GI-diseases, GI-infections, and allergies
- to ensure the stability and viability of probiotic products by developing feasible technologies (e.g. process and material development for microencapsulation).
- to develop technology for non-dairy, novel or artificial probiotic applications.
- to evaluate the role of probiotics in healthy consumer groups and to address consumer aspects

One of the areas of most active research pertains to the mechanism of action of probiotics. Current probiotics have mainly been selected based on the common criteria (resistance to pancreatic enzymes, acid and bile, adhesion to the intestinal mucosa, human origin, documented health effects, safe, good technological properties). To refine the selection criteria, understanding of the mechanisms of probiotic action is necessary. The interaction between a probiotic strain and cocktail of strains/species must be examined in the context of those interactions that normally take place between the microbiota and the host, as well as between individual components of the microbiota. This will make it possible to select future stains with more specific characteristics, to suit the needs of specific age and patient groups. This need is clearly indicated by the difference in mucosal adhesion of probiotic bifidobacteria to mucus of different age groups and the influence of disease on mucosal adhesion of selected probiotics. The use of specially selected probiotics for particular subject groups may provide more specific health effects (Ouwehand *et al.*, 2002; Quigley, 2010).

Good viability and activity of probiotics are considered prerequisites for optimal functionality. However, several studies have shown that non-viable probiotics can have beneficial effects such as immune modulation and carcinogen binding in the host. Thus, for certain probiotic strains it might be sufficient that they grow well during initial production steps (to obtain high enough cell numbers in the product) but they do not necessarily need to retain good viability during storage. Non-viable probiotics would have several advantages over viable ones: longer shelf life, improved safety and no need for refrigerated storage or transport (Mattila-Sandholm, 2002; Ouwehand *et al.*, 2002).

Current industrial probiotic foods are basically dairy products, which may represent inconveniences due to their lactose and cholesterol content (Rivera-Espinoza and Gallardo-Navarro, 2010). Application of probiotic cultures in non-dairy products and environments represents a great challenge. Probiotic viability in the food matrix depends on factors such as pH, storage temperature, oxygen levels, and presence of competing microorganisms and inhibitors. Storage at room temperature, which is common for many types of non-dairy products, such as cereal products, drinks, confectionary etc., can create an overwhelming challenge for probiotic stability (Mattila-Sandholm, 2002).

Technological advances have made possible to alter some structural characteristics of fruit and vegetables matrices by modifying food components in a controlled way. This could make them ideal substrates for the culture of probiotics, since they already contain beneficial nutrients such as minerals, vitamins, dietary fibers, and antioxidants (Rivera-Espinoza and Gallardo-Navarro, 2010). Beverages would be the next food category where the healthy bacteria will make their mark. Likely candidates are chilled fruit juices, bottled water, or fermented vegetable juices. The probiotic microorganisms also have been directly incorporated into the drinks. Adding probiotics to the juices is more complex than formulating in the dairy products because the bacteria need protection from the acidic conditions in the fruit juice. However, with microencapsulation technologies, the probiotics can become an important ingredient in the functional foods (Prado *et al.*, 2008; Chávari *et al.*, 2010). Meat has been shown to be an excellent vehicle for probiotics. The buffering capacity of meat may be due to a raised pH of the microenvironment of bacteria living on the surface. Furthermore, meat has been found to protect LAB against the lethal action of bile. The target products in meat processing are the various dry sausages that are processed by fermenting without heating (Rivera-Espinoza and Gallardo-Navarro, 2010).

IV.2.2. Lactic Acid Bacteria as live vaccines

Edible vaccines are mucosal-targeted vaccines, which cause stimulation of both systematic and mucosal immune response (Mishra *et al.*, 2008). Mucosal routes for vaccine delivery offer several advantages over systemic inoculation by minimizing potential adverse effects and by the ease of administration. One way to deliver protective antigens at mucosal surfaces is to use live bacterial vectors. Over the last two decades, the use of recombinant bacteria as carrier system to deliver antigens to the mucosal immune system has been widely investigated. Most strategies have relied on the use of attenuated pathogenic bacteria, among which is the use of invasive but non-pathogenic *Salmonella*. A number of other bacteria such as *Listeria*, *Vibrio*, *Bordetella*, *Mycobacterium* have also been proposed, although the major concern in the use of attenuated pathogens is that they may still be virulent in the elderly and in very young children. Food LAB usage would overcome this problem since these bacteria have a long history of safe use and could possibly be delivered safely at a high dose (Renault, 2002).

Non-pathogenic food grade bacteria, such as LAB, are being tested for their efficacy as live antigen carriers. The LABVAC European research network is presently comparing the vaccine potential of *Lactococcus lactis*, *Streptococcus gordonii* and *Lactobacillus* spp. (Mercenier *et al.*, 2000). More specifically, *Lactococcus lactis* is a potential candidate for the production of biologically useful proteins. A promising application is its use as an antigen delivery vehicle for the development of live mucosal vaccines (Anderson *et al.*, 2010). Use of these live bacteria to elicit an immune response or to carry a vaccine component is a new invention in vaccine development. The advantage of live bacterial vaccines is that they mimic natural infection, have intrinsic adjuvant properties and can be given orally (Amdekar *et al.*, 2010). One of the advantages of oral vaccines is that they stimulate production of mucosal antibodies more effectively than is the usual case with injectable vaccines (Korban *et al.*, 2002).

Two strategies should be followed in the future.

- laboratory production systems based on antibiotic resistance genes should be replaced by food-grade systems much better accepted by potential consumer
- cocktails should be used of recombinant lactococci producing antigens and cytokines and lactobacilli as probiotic adjuvants (Anderson *et al.*, 2010)

IV.2.3. Genetically modified microorganisms

The use of recombinant DNA technology to produce genetically modified microorganisms is one of the most important scientific advances of the 20th century. It has great potential in research because it allows the development of highly sensitive analytical procedures. It also has potential in industry, leading to processes and products that would be difficult to develop using conventional techniques (Henriksen *et al.*, 1999). By means of genetic engineering, the properties of microorganisms, e.g. starter cultures, can be changed more precisely than by random mutagenesis and subsequent selection procedures based on classical bacteriological and genetic methods. Major goals are optimization of the production process, improvement of product quality and safety (hygienic status) and enlargement of product diversity. The broad spectrum of fermentation processes applied in food production and the important role of microorganisms in functional foods (e.g. probiotics) demonstrate the enormous potential of genetic engineering for improving microorganisms used in food industry (Engel *et al.*, 2002; Sybesma *et al.*, 2006).

Although various strains of food bacteria have been improved by genetic engineering there is still very limited application of these GMO strains. Main problems are the lack of robust procedures for risk assessment, the lack of knowledge on horizontal gene transfer and on undesired side effects of genetic modifications, and on the unpredictability of the effect of spreading the modified bacteria in nature, e.g., after passage through a human being. Many efforts have been made in the field of developing tools for self-cloning, to ensure that no heterologous DNA is present in the GMO to be applied to food fermentation or as a probiotic. Novel methods are also being developed for generation of food-grade genetically modified bacteria by site-specific recombination, including site-specific DNA resolution of nonfood-grade DNA used during the construction. Use of genomic tools such as DNA microarrays can contribute to devising robust risk assessment procedures, which are needed by

food manufacturers and consumers. These and other developments will facilitate food applications of GMOs with improved industrial properties, but even more importantly, with clear consumer benefits (Kuipers *et al.*, 2000).

The use of genetically modified microorganisms in food and food processing is controversial due to a lack of acceptance by consumers, especially in Europe (Henriksen *et al.*, 1999). In every case, related to the use of GMM in food production various aspects are considered including the strategies used for the construction of strains with respect to current legislation requirements, the environmental risk evaluations concerning the deliberate release of genetically modified microorganisms, the methods for detection of recombinant DNA and protein that are currently under evaluation, and the reasons behind the critical public perception towards the application of such strains (Schuller and Casal, 2005).

IV.3. Main gaps in terms of knowledge, technologies and policies

IV.3.1. Gaps in knowledge

A food ecosystem is not static. The dynamics of growth, survival and biochemical activity of microorganisms in foods are the result of stress reactions in response to changing physical and chemical conditions that occur in the food micro-environment, the ability of microorganisms to colonize the food matrix and to grow into spatial heterogeneity, and the *in situ* cell-to-cell ecological interactions, which often happen in a solid phase. Reliable quantitative microbiological data should, therefore, take into consideration the dynamics of microorganisms in food ecosystems. Information on these dynamics is of key importance in food ecology, especially in understanding the behavior of microorganisms in foods (Giraffa and Carminati, 2008).

Throughout the world there are many different types of fermented foods, in which a range of different substrates are metabolized by a variety of microorganisms to yield products with unique and appealing characteristics. In many of these foods, the biological and microbiological bases of the fermentation processes are poorly understood. What little information is available often deals with the identification and perhaps preliminary characterization of the primary microbiota in the finished product. In some instances, there will undoubtedly be a need in the future to produce these foods in circumstances where quality and safety can be guaranteed. This in turn will necessitate a more thorough understanding of the microorganisms involved, in terms of the types and their specific activities, so that the fermentation process can be made more reliable and predictable. One of the challenges facing scientists and technologists in the future will undoubtedly be to allow the large-scale production of fermented foods without losing the unique flavour and other traits associated with the traditional products from which they are derived (Caplice and Fitzgerald, 1999).

Among future challenges for food science in the field of more efficient improvement of microorganisms used in food processing is the better understanding of factors affecting the establishment, survival and growth of microorganisms by multidisciplinary research on physiology of microorganisms, development of predictive models for their growth and survival, effect of food structure on its interactions with microorganisms and *vice-versa* and application of molecular biology for improved characterization, typing and detection of important microorganisms (Farkas, 2001). The mathematical analysis of the biokinetics of beneficial microorganisms represents a quite novel, but very promising and rich domain of research. Strains that show excellent functional behaviour under optimal laboratory conditions frequently fail once they are applied in the food matrix. Specific environmental conditions that characterize a food environment thoroughly interfere with bacterial cell metabolism. In many cases, distinct information about this interference is lacking due to the complexity of the food matrix. Predictive modelling may yield precious information about the relationship between the food environment and bacterial functionality, and may hence contribute to optimal strain selection and process design. This may result in better process control, enhanced food safety and quality, and reduction of economic losses (Leroy *et al.*, 2002).

For those fermentations where there already exists a considerable body of knowledge regarding the role and activity of the relevant microbiota, the challenges enfacing the scientists and technologists are somewhat different. Here, the goals are to further improve reliability and product quality through

optimization of starter culture performance and to eliminate those factors that impede the fermentation process (Caplice and Fitzgerald, 1999).
In conclusion, we are now entering the post-genomic age of microbiology at a time when many microorganisms used for food production have already been sequenced. This offers a new knowledge-based approach to the exploitation of bacteria for food production, from metabolic engineering of microorganisms to produce antimicrobials or nutritionals, to the molecular mining of activities as yet unknown but which could benefit food production. The real challenge of the genomics era, as it applies to food systems, is the harnessing of this wealth of information for improved culture performance and activities, thereby improving the safety and quality and composition of our food supply (Paul Ross et al., 2002).

IV.3.2. Gaps in technology

Knowledge about microbial community composition, its different populations and its potential interactions in food-associated matrices is crucial to ensure production of safe and high-quality food (Justé *et al.*, 2008).

A promising tool for the advancement of studies on food-associated microbial populations, either cultivable or not cultivable, is the application of real-time quantitative PCR (qPCR) to food systems, which is considered as a method of choice for the detection and quantification of microorganisms. However, the use of RT-qPCR to study changes in growth and metabolic activities of microbial populations is only beginning. Further development in this field should provide useful information to control organoleptic characteristics during fermented food product making and for microbial risk assessment during industrial processes. In the near future several interesting applications of RT-qPCR may be considered, such as studying the impact of different steps of industrial processes on the expression of target genes. The range of applications could take place at all stages, from starter cultures to conservation and storage of the final product. The detection and quantification of transcripts predicting for the presence of undesirable molecules and risk analysis are also interesting applications, as suggested in a few recent articles (Giraffa and Carmintati, 2008; Postollec *et al.*, 2011).

The DNA chip microarray technology is a direct result of the availability of genome sequence information. DNA chips can be used for simultaneous monitoring of levels of expression of all of the genes in a cell, in order to study whole genome expression patterns in various matrices during development. Moreover, since parallel hybridisations of hundreds or thousands of genes in a single experiment can be performed by high throughput DNA microarrays, direct profiling of microbial populations is achievable. The hybridisation of bulk DNA extracted from food to chip-bound probes is a promising tool for microbial community analyses in foods. In one development of this basic technique, genome-probing microarrays (GPMs) were developed by depositing hundreds of microbial genomes as labeled probes on a glass slide, and by their hybridisation with bulk community DNAs. GPM enabled quantitative, high-throughput monitoring of LAB community dynamics during fermentation of kimchi, a traditional Korean food. Compared to currently used oligonucleotide microarrays, the specificity and sensitivity of GPM was remarkably increased to a species-specific level (Bae *et al.*, 2005; Giraffa and Carmintati, 2012).

Among the methods to gain access to the genetics and physiology of uncultured organisms, metagenomics, the genomic analyses of a population of microorganisms, has emerged as a powerful centre-piece. The ability to clone large fragments of metagenomic DNA allows entire functional operons to be targeted with the possibility of recovering entire metabolic pathway. Constructing metagenomic libraries captures the phylogenetic and genetic diversity in environmental samples. The genetic potential of the libraries has only begun to be tapped. The small molecules and enzymes already discovered indicate the potential of metagenomics to mine the environment for fundamental knowledge and products for biotechnology. The generation and analysis of metagenomic libraries is a powerful approach to harvest and archive environmental genetic resources. It could be useful to identify which organisms are present in food ecosystems, what they do, and how their genetic information can be beneficial to mankind. Future advances in understanding the differences among communities or environments will be derived from “comparative metagenomics” in which libraries

prepared from different sites or at different times can be compared (Randazzo *et al.*, 2009; Riesenfeld *et al.*, 2004).

Metagenomics and metatranscriptomics approaches can be very powerful for gaining important insights in fermented foods. For example, in defining cultivation conditions for so far uncultured bacteria, these approaches can provide information about the critical fermentation parameters affecting quality and information about interactions, and possibly trophic chains, between bacteria within these fermentation ecosystems. Moreover these approaches will be propelled by the tremendous advances in sequencing, since the latest sequencing technologies are increasingly used to study the phylobiomes and genomes of bacteria in traditional food and beverage fermentations, with pyrosequencing often referred as the metagenome or the sequencing method currently used. More widespread application of bioinformatics tools as well as time spent on in-depth analysis and interpretation of the data generated will maximize the insights derived from such studies (van Hijum *et al.*, 2012).

IV.3.3 Gaps in policies

Because of the legislative framework, an issue brought up by some industrialised states is that the high level of scientific approach in Europe will hamper or jeopardize innovation especially regarding functional foods.

Functional foods are closely associated with claims on foods. Regulation 1924/2006 of the European Union identifies two categories of claims on foods: nutrition claims and health claims. Nutrition claims are claims that state, suggest or imply that a food has particular beneficial nutritional properties due to the energy it provides or the nutrients it contains while health claims are claims that state, suggest or imply a relationship between a food or food category and health. Health claims on (functional) foods must be scientifically substantiated. As concerns scientific evaluation, the EU-project "Process for the Assessment of Scientific Support for Claims on Foods (PASSCLAIM)" resulted in a set of criteria for the scientific substantiation of health claims on foods. The PASSCLAIM project engaged more than 160 scientists from academia, industry, research institutes, public interest groups, and the regulatory environment. The project defined a number of generally applicable criteria for the scientific support of claims. These criteria emphasized the need for direct evidence of benefits to humans, recognized the usefulness of markers of intermediate effects, and emphasized that effects should be both statistically and biologically meaningful. The European Food Safety Authority (EFSA) provides the scientific advice to the European Commission for health claims submitted under Regulation 1924/2006 and has *hitherto* published several hundreds of opinions on health claims (Asp and Bryngelsson, 2008; Verhagen *et al.*, 2010). The rejection by EFSA of at least 74 submissions for probiotic health claims, even after resubmission, is also referred to under Section II. 3.a., when addressing "impeding factors".

As for nutrition claims the Regulation stipulates that such claims are only permitted if they are listed in its annex, and to be sure, fulfill the conditions laid down in the Regulation. Producers and/or Member States who would like to use a specific nutrition claim not on the list should therefore consult with the Commission who has to power to amend the annex of Regulation 1924/2006. In cases where Member States representatives do not agree with the Commission's view, the Council and/or the European Parliament may oppose to the Commission adopting a decision. As regards health claims, there are different procedures to be followed. For Article 14 claims, the reduction of disease risk claims and claims referring to children's development and health, the European Commission will formally need to authorize all individual applications for health claims, which need to be scientifically substantiated. An application needs to be submitted through an appropriate national food authority, who will then forward the application to EFSA. EFSA will give a scientific advice on the substantiation of the health claim and send this to the European Commission. The Commission will decide whether or not to authorize the application for a health claim on the basis of the regulatory committee procedure with scrutiny. For health claims according to Article 13.1 (function claims), a different procedure applies. Based on the EFSA opinions, the European Commission will establish a list of positive health claims in accordance with the regulatory committee procedure with scrutiny. Health claims thus approved, can subsequently be used by producers without recurrence to the European Commission, provided that they are in conformity with the various legal requirements set (Verhagen *et al.*, 2010).

In the USA, Japan and Codex Alimentarius different rules apply. In the USA, claims on food and dietary supplement labels fall into three categories: nutrient content claims, structure/function claims and health claims. These are quite similar in nature to their European equivalent, albeit that their Regulatory status and procedures for use and approval is very different. In the USA, ‘‘qualified health claims” (QHC) are legally allowed under the provision that they carry a disclaimer to indicate the lower level of scientific evidence (Verhagen *et al.*, 2010). Those claims are not substantiated on evidence that meets the level of Significant Scientific Agreement standard (SSA), but include a qualifying statement intended to convey to the consumer the level of evidence for the claim (Rowlands and Hoadley, 2006). In Japan, the ‘‘foods for specific health use” (FOSHU) law only requires some evidence (Verhagen *et al.*, 2010). The current Japanese ‘‘Food with Health Claims’’ include 2 categories. For the first category, ‘‘Food with Nutrient Function Claims,’’ the label may be freely used if a product satisfies the standard for the minimum and maximum levels per daily portion usually consumed. The second category is defined as ‘‘Food for Specified Health Uses’’ (FOSHU). FOSHU foods are those that contain dietary ingredients that have beneficial effects on the physiological functions of the human body, maintain and promote health, and improve health-related conditions. Any manufacturer who applies to the government for approval under the FOSHU code for its product must tabulate both published available publications and internal reports on the effectiveness of the product and/or its ingredients and provide a summary of each available publication or report. The tabulation must include in vitro metabolic and biochemical studies, in vivo studies, and randomized controlled trials on Japanese people (Yamada *et al.*, 2008).

Whereas the Japanese and USA have quite liberal regulatory provisions for health claims, Europe displays requirements for a high level of scientific substantiation as reflected in the Regulation 1924/2006 and widely supported by scientists in Europe. The scientists in PASSCLAIM also acknowledge that grading of evidence is a political/ policy rather than scientific issue. However some appeals have been made towards a more liberal system in Europe and more in line with the USA and Japan (Verhagen *et al.*, 2010).

Another issue arising related to legislation concerns the use of genetically modified (GM) microorganisms in food production. The United States bases its regulations about GM food on end products, without considering the methodology used for its production and thus substantially equivalent GM food is subject to the same basic regulations as other foods. Canada's regulations are also product based, but Canada also treats all GM food as "novel". It therefore requires GM foods to pass a pre-marketing safety assessment, but then approved GM food is considered conventional and subject to the same laws as traditional foods. Most other countries base their regulations on the production process and regard biotech food as novel, requiring a specific set of approval and labeling regulations. The regulatory framework in Canada and the United States respects the scientific consensus that was in place at the beginning of the 1990s, which states that foods produced with biotechnology do not necessarily present any new or greater risk than traditional foods. This approach is based on the concept of *substantial equivalence.* The safety reviews are for the purposes of determining that the GM food is equivalent to traditional foods in terms of risk. On the other hand, the EU has built its safety system under the premise that GM food may present specific risks and thus should be treated separately from conventional foods. The EU approval procedures for GM food are based on the *precautionary* principle. This approach postulates that biotech food may contain unknown risks, and EU countries believe they should take appropriate measures to limit the development of future unknown risks to human health and the environment. The Cartagena Protocol on Biosafety, which counts more than 100 ratifying countries, is also based on process-based standards (it aims to regulate the movement of living modified organisms, which include GM crops, GM seeds, and GM food) and the precautionary principle (Carter and Gruère, 2006).

IV.4. Priority actions/conclusions and recommendations

The role and diverse applications of beneficial microorganisms in food processing have been addressed in this study. With the major focus on the status and trends of their conservation and sustainable use, key information was summarised and major issues, also with regard to expected future developments and needs, were outlined.

With numerous on-going activities, and availability of detailed information on the use of

microbial interactions in food processing, there seems to be a **need for better coordination** and exchange, both at the national and international levels. One approach may be directed to the improvement of the quality and safety of food produced by traditional, "low-tech" processes. Instead of a "top-down" approach, an important step should be to create a common forum for all stakeholders, structured to incorporate local/regional and national "platforms" or fora. Centralised, global coordination by an international NGO could help to link and integrate actions of these platforms. Activities may include:

- Facilitating and encouraging participation of all stakeholders.
- Exchange of general, scientific and technical information.
- Facilitating access to specialised technical information on food processing biotechnology by promoting knowledge transfer between the public and private sectors.
- Organising of workshops and other opportunities for training and education.
- Giving guidance on key issues and attending to concerns of small-scale processors.
- Opening ways and means towards un-bureaucratic, low-cost access to microbial strains (in strain collections, both in the formal and informal sectors) suitable for technical use in small-scale operations.
- Enabling communication and exchange between local and central governments on the one side, and small-scale stakeholders, on the other side.
- Providing guidance and support to governments on the application of food processing biotechnologies and on their role and importance in food safety and security.
- Providing technical advice and facilitate access to science park and technology business incubator governance. Initiated by UNESCO-WTA, these economic platforms support start-up companies to grow into mature businesses by providing resources such as property services and physical infrastructures for business and research, venture capital and legal support.
- Supporting the dissemination of scientific and technical information generated by collaborative research projects, e.g., in the EU within STD, INCO and other programmes.
- Developing a comprehensive global database in which relevant information on nutritional, health and health-benefit claims associated with fermented foods is collected and organised. This should also be backed up by other available data (from scientific literature, internal reports and unpublished documents) on beneficial aspects of fermented foods.
- Organising and facilitating an international network for all stakeholders with links to regional networks. Information can be shared on (e.g.):
    - ✓ Opportunities for collaborative research and development (R&D),
    - ✓ Access to Science Parks and other institutions,
    - ✓ Practical experiences,
    - ✓ Documents and reports on topics relevant to different levels of food processing biotechnologies.

A "multiplication" approach has huge potential for wide-range dissemination to small-scale processors, and may encompass, e.g., the "**training of trainers**". Training and preparing trainers on specific needs, questions and concerns of small-scale stakeholders would have numerous benefits. These trainers can:

- Constitute a vital link between the formal and informal sectors.
- Interact with national and international platforms (fora).
- Provide technical assistance and guidance on key issues (critical steps in processing; the basic principles of GHP, GMP, etc., basic equipment for packaging and storage; availability and selection of raw materials; approaches towards using appropriate starter cultures vs. "back-slopping"; marketing approaches, etc.) to small-scale processors.
- Support and guide the promotion of traditional fermented foods on the local, regional and

national levels.

The following actions are considered to have high priority and are recommended for inclusion in planning of future policies:

- Facilitating and encouraging intensified studies on traditional food fermentations with the purpose of:
    - ✓ Characterising the microbial population diversity,
    - ✓ Identifying strain/species domination, including the key "role players" in terms of typical quality attributes to the final product,
    - ✓ Selecting and facilitating the depositing of appropriate strains for the development of starter cultures.

- Introducing starter cultures for small-scale food fermentations. The benefits of starter cultures for traditional small-scale fermentations have long been realised. This approach has been identified by the FAO as a high priority towards improving the quality and safety of these products. By applying starter cultures, metabolic activities will be accelerated and fermentation processes will be more predictable and better controlled. Important advantages will include safety improvement and reduction of hygienic and toxicological risks. However, the general introduction of low-cost starter cultures for small-scale operations has not been realised yet. Constraints such as cost, handling and distribution, and technical limitations and logistics, are major factors that have hampered this development thus far. However, valuable examples of successful introduction of low-cost starters have been documented and may serve as models for future developments. An example of the successful application of low-cost concentrated starter cultures for cassava fermentation by a women's enterprise in Benin, has been discussed in Section 2.3.

- In support of starter culture development, a research and infrastructural support base needs to be established and strengthened. This should also take into account bioreactor design and the development of diagnostic equipment for monitoring of starter culture performance at each level. Basic laboratory equipment and cell bank facilities should form part of the infrastructure enabling preservation and storage of microbial culture preparations. Pilot processing facilities are required to enable scaling up of application levels towards adoption of a strain or mixed culture.

- Promoting small-scale starter culture processing in rural areas by making use of "low-tech" procedures, and supporting local networking among starter providers and small-scale processors. This may comprise (amongst others):
    - ✓ The development and implementation of simple but effective methods for preserving and maintaining traditional starter cultures, requiring no refrigeration but with extended shelf-life under ambient conditions.
    - ✓ Consideration of the microbial biodiversity for assuring the typical traits of a traditional fermented food product.
    - ✓ Comparing the suitability of different carrier materials or substrates for immobilising and preserving starters.
    - ✓ The further development and standardisation of traditional methods to withstand climatic fluctuations.

- In view of the potential effects of climate change and the alarming rate of desertification, the

development of mathematical models for food fermentations seems imperative. These models should be designed for predicting the behaviour of microbial communities typical of traditional fermented foods of these regions, relative to the potential impact of climate change.

- On the basis of preservation mechanisms in food fermentations, further development and applications of consumer-friendly, "natural" processing approaches such as bio-preservation should be promoted as alternative to chemical and/or thermal preservation (discussed in Section 2.3).

- Promoting the increased use of modern "omics" approaches as powerful tools by which important insights in fermented foods may be gained.

- Intensification of studies on functional properties of traditional fermented foods by which health promoting (probiotic) effects may be identified. The application of "functional genomics" for strain characterisation can be a valuable tool in support of these studies, and in the selection of beneficial strains. Making better use of existing opportunities offered by the EU and other Organisations, for capacity building and training, e.g., by workshops, seminars and conferences, and also by participating in research projects. Training and guidance should also be directed towards successful project applications. This may include support in networking and access to potential project partners.

- While organisations such as FAO may not be directly involved in the regulation of health related claims, they may still contribute and share experience and knowledge. By doing so, they would be contributing towards a more open and well regulated approach for approval of health-related claims by which the benefits of fermented foods are substantiated. The classical example worthwhile considering is based on an approach introduced by the Japanese government involving an approval process for functional foods, called "Foods for Specified Health Use" (FOSHU) in the 1980s. Compared to only one formal approval by EFSA of a probiotic health claim (alleviation of lactose tolerance) as from December 2012, around 955 foods (including probiotics) have been approved for FOSHU by the Consumer Affairs Agency of the Japanese Ministry of Health, Labour and Welfare by 2011. This approval process requires a detailed review process with scientific evidence for each application. The CODEX Committees on Food Labelling and on Nutrition and Foods for Special Dietary Uses (Codex Alimentarius) are participating in the establishment of Nutrition Labelling Standards in Japan. These activities are coordinated by the WHO Global Strategy on Diet, Physical Activity, and Health, and are an example of successful controlled regulation of the promotion of "health foods".

- Reinforcing and/or adapting the Convention on Biological Diversity, especially in developing countries, so that their sovereign rights over their natural resources are recognised. The Nagoya Protocol (yet to enter into force) may serve in this respect, as it addresses (a) traditional knowledge associated with genetic resources with provisions on access, benefit-sharing and compliance, and (b) genetic resources where indigenous and local communities have the established right to grant access to them.

ANNEXES

❖

ANNEX 1

Uses of microorganisms in food processes, including in traditional food processing methods

❖

ANNEX 2

CASE STUDY

Asia: Himalayan traditional fermented foods

❖

ANNEX 3

Examples of genetically engineered microorganisms and their applications in food processes

ANNEX 1
Uses of microorganisms in food processes, including in traditional food processing methods

TABLE I

Main types of microorganisms and their specific uses in food processing

Group / Genera / Species	Major application(s)	Reference
Bacteria		
Acetobacter		
Acetobacter aceti subsp. aceti	Vinegar	Bourdichon *et al.*, 2011
Acetobacter fabarum	Cocoa, Coffee	Bourdichon *et al.*, 2011
Acetobacter lovaniensis	Vegetables	Bourdichon *et al.*, 2011
Acetobacter malorum	Vinegar	Bourdichon *et al.*, 2011
Acetobacter orientalis	Vegetables	Bourdichon *et al.*, 2011
Acetobacter pasteurianus subsp. pasteurianus	Rice vinegar, Cocoa	Sengum and Karabiyikli, 2011
Acetobacter pomorum	Industrial vinegar	Sengum and Karabiyikli, 2011
Acetobacter suboxidans	Ascorbic acid (food additive)	Rani and Soni, 2007
Acetobacter syzygii	Vinegar, Cocoa	Bourdichon *et al.*, 2011
Acetobacter tropicalis	Cocoa, Coffee	Bourdichon *et al.*, 2011
Arthrobacter		
Arthrobacter arilaitensis	Dairy	Bourdichon *et al.*, 2011
Arthrobacter bergerei	Dairy	Bourdichon *et al.*, 2011
Arthrobacter globiformis	Citrus fermentation to remove limonin and reduce bitterness	Mogensen *et al.*, 2002b
Arthrobacter nicotianae	Cheese maturation	Mogensen *et al.*, 2002b
Bacillus		
Bacillus acidopulluluticus	Pullulanases (food additive)	Rani and Soni, 2007
Bacillus amyloliquefaciens	Fish	Bourdichon *et al.*, 2011
Bacillus coagulans	Cocoa[1] Glucose isomerase (food additive)[2]	Bourdichon *et al.*, 2011[1]; Rani and Soni, 2007[2]
Bacillus licheniformis	Protease (food additive)	Rani and Soni, 2007
Bacillus pumilus	Locust beans	Ouoba *et al.*, 2004
Bacillus subtilis	Soy[1], Protease, Glycolipids, Riboflavin-B_2 (food additive)[2]	Bourdichon *et al.*, 2011[1]; Rani and Soni, 2007[2]
Bifidobacterium		
Bifidobacterium adolescentis	Used in fermented milks Probiotic properties	Mogensen *et al.*, 2002b
Bifidobacterium animalis subsp animalis	Used in fermented milks Probiotic properties	Mogensen *et al.*, 2002b
Bifidobacterium animalis subsp lactis	Fermented milks with probiotic properties Common in European fermented milks	Mogensen *et al.*, 2002b
Bifidobacterium bifidum	Used in fermented milks as probiotic ingredient	Mogensen *et al.*, 2002b

Bifidobacterium breve	Used as probiotics in fermented milks and infant formulas Soy	Mogensen *et al.*, 2002b
Bifidobacterium longum	Fermented milks with probiotic properties	Bourdichon *et al.*, 2011
Bifidobacterium pseudolongum subsp pseudolongum	Fermented milk and probiotic for animals	Mogensen *et al.*, 2002b
Bifidobacterium thermophilum	Dairy	Bourdichon *et al.*, 2011
Brachybacterium		
Brachybacterium alimentarium	Gruyère and Beaufort cheese	Bourdichon *et al.*, 2011
Brachybacterium tyrofermentants	Gruyère and Beaufort cheese	Bourdichon *et al.*, 2011
Brevibacterium		
Brevibacterium ammoniagenes	Nucleosides (food additive)	Rani and Soni, 2007
Brevibacterium aurantiacum	Used for cheese production	Bourdichon *et al.*, 2011
Brevibacterium casei	Used for cheese production	Mogensen *et al.*, 2002b
Brevibacterium flavum	Malic acid, Glutamic acid, Lysine, Monosodium glutamate (food additives)	Rani and Soni, 2007
Brevibacterium linens	Soft cheese ripening	Bockelmann *et al.*, 2005
Carnobacterium		
Carnobacterium divergens	Dairy, Meat, Fish	Bourdichon *et al.*, 2011
Carnobacterium maltaromaticum	Dairy, Meat	Bourdichon *et al.*, 2011
Carnobacterium piscicola	Meat	Bourdichon *et al.*, 2011
Corynebacterium		
Corynebacterium ammoniagenes	Cheese ripening	Mogensen *et al.*, 2002b
Corynebacterium casei	Cheese ripening	Bourdichon *et al.*, 2011
Corynebacterium flavescens	Used in cheese ripening cultures	Mogensen *et al.*, 2002b
Corynebacterium glutamicum	Glutamic acid, Lysine, Monosodium glutamate (food additives)	Rani and Soni, 2007
Corynebacterium manihot	Cassava root	Caplice and Fitzerald, 1999
Corynebacterium variabile	Cheese ripening	Bourdichon *et al.*, 2011
Enterobacter		
Enterobacter aerogenes	Bread fermentation	Mogensen *et al.*, 2002b
Enterococcus		
Enterococcus durans	Cheese and sour dough fermentation	Mogensen *et al.*, 2002b
Enterococcus faecalis	Dairy, Meat, Soy, Vegetables	Bourdichon *et al.*, 2011
Enterococcus faecium	Dairy, Meat, Soy, Vegetables	Bourdichon *et al.*, 2011
Gluconacetobacter		

Gluconacetobacter azotocaptans	Cocoa, Coffee	Bourdichon *et al.*, 2011
Gluconacetobacter diazotrophicus	Cocoa, Coffee	Bourdichon *et al.*, 2011
Gluconacetobacter entanii	Vinegar	Bourdichon *et al.*, 2011
Gluconacetobacter europaeus	Vinegar	Bourdichon *et al.*, 2011
Gluconacetobacter hansenii	Vinegar	Bourdichon *et al.*, 2011
Gluconacetobacter johannae	Cocoa, Coffee	Bourdichon *et al.*, 2011
Gluconacetobacter oboediens	Vinegar	Bourdichon *et al.*, 2011
Gluconacetobacter oxydans	Vinegar	Bourdichon *et al.*, 2011
Gluconacetobacter xylinus	Vinegar	Bourdichon *et al.*, 2011
Hafnia		
Hafnia alvei	Ripening of meat Dairy	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
Halomonas		
Halomonas elongata	Ripening of ham	Mogensen *et al.*, 2002b
Klebsiella		
Klebsiella aerogenes	Tryptophan (food additive)	Rani and Soni, 2007
Kocuria		
Kocuria rhizophila	Dairy, Meat	Bourdichon *et al.*, 2011
Kocuria varians	Dairy, Meat	Bourdichon *et al.*, 2011
Lactobacillus		
Lactobacillus acetototolerans	Ricotta cheese, Vegetables	Bernardeau *et al.*, 2006; Bourdichon *et al.*, 2011
Lactobacillus acidifarinae	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus acidipiscis	Dairy, Fish	Bourdichon *et al.*, 2011
Lactobacillus acidophilus	Fermented milks, Probiotics, Vegetables	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
Lactobacillus alimentarius	Fermented sausages Ricotta Meat, Fish	Mogensen *et al.*, 2002b; Bernardeau *et al.*, 2006
Lactobacillus amylolyticus	Bread fermentation Production of glycoamylase	Mogensen *et al.*, 2002b
Lactobacillus amylovorus	Bread fermentation Production of glycoamylase	Mogensen *et al.*, 2002b
Lactobacillus bavaricus	Meat fermentation and biopreservation of meat	Mogensen *et al.*, 2002b
Lactobacillus brevis	Bread fermentation Wine Dairy, Vegetables	Mogensen *et al.*, 2002b; Bourdichon et al., 2011 Bernardeau *et al.*, 2006;

Lactobacillus buchneri	Malolactic fermentation in wine Sourdough	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
Lactobacillus cacaonum	Cocoa	Bourdichon *et al.*, 2011
Lactobacillus casei subsp casei	Dairy starter Cheese ripening Green table olives	Mogensen *et al.*, 2002b; Bernardeau *et al.*, 2006
Lactobacillus collinoides	Wine Fruits	Bernardeau *et al.*, 2006; Bourdichon *et al.*, 2011
Lactobacillus composti	Beverages	Bourdichon *et al.*, 2011
Lactobacillus coryniformis subsp coryniformis	Fermentation of cheese and cassava	Mogensen *et al.*, 2002b
Lactobacillus crispatus	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus crustorum	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus curvatus subsp curvatus	Meat	Bourdichon *et al.*, 2011
Lactobacillus delbruecki subsp. bulgaricus	Yogurt and other fermented milks Mozarella	Mogensen *et al.*, 2002b; Bernardeau *et al.*, 2006
Lactobacillus delbruecki subsp. delbruecki	Fermented milks, Vegetables	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
Lactobacillus delbruecki subsp. lactis	Fermented milk and cheese	Mogensen *et al.*, 2002b
Lactobacillus dextrinicus	Meat	Bourdichon *et al.*, 2011
Lactobacillus diolivorans	Cereals	Bourdichon *et al.*, 2011
Lactobacillus fabifermentants	Cocoa	Bourdichon *et al.*, 2011
Lactobacillus farciminis	Fermentation of bread Soy, Fish	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
Lactobacillus fermentum	Fermented milks[1], Sourdough[2] Urease (food additive)[3]	Mogensen *et al.*, 2002b[1]; Bourdichon et al., 2011[2]; Rani and Soni, 2007[3]
Lactobacillus fructivorans	Beverages	Bourdichon *et al.*, 2011
Lactobacillus frumenti	Cereals	Bourdichon *et al.*, 2011
Lactobacillus gasseri	Fermented milk and probiotics Ricotta Sourdough	Mogensen *et al.*, 2002b; Bernardeau *et al.*, 2006
Lactobacillus ghanensis	Cocoa	Bourdichon *et al.*, 2011
Lactobacillus hammesii	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus harbinensis	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus helveticus	Starter for cheese Cheese ripening Vegetables	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
Lactobacillus hilgardii	Malolactic fermentation of wine	Mogensen *et al.*, 2002b

Lactobacillus homohiachii	Beverages, Sourdough	Bourdichon *et al.*, 2011
Lactobacillus hordei	Beverages	Bourdichon *et al.*, 2011
Lactobacillus jensenii	Fermentation of cereals	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
	Sourdough	
Lactobacillus johnsonii	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus kefiri	Fermented milk (Kefir)	Bourdichon *et al.*, 2011
	Reduction of bitter taste in citrus juice	
Lactobacillus kefranofaciens subsp kefiranofaciens	Dairy	Bourdichon *et al.*, 2011
Lactobacillus kefranofaciens subsp kefirgranum	Dairy	Bourdichon *et al.*, 2011
Lactobacillus kimchii	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus kisonensis	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus mali	Wine	Bernardeau *et al.*, 2006
	Fruits	Bourdichon *et al.*, 2011
Lactobacillus manihotivorans	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus mindensis	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus mucosae	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus nagelii	Cocoa	Bourdichon *et al.*, 2011
Lactobacillus namurensis	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus nantensis	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus nodensis	Dairy	Bourdichon *et al.*, 2011
Lactobacillus oeni	Wine	Bourdichon *et al.*, 2011
Lactobacillus otakiensis	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus panis	Sourdough, bread	Bourdichon *et al.*, 2011
Lactobacillus parabrevis	Dairy, Vegetables	Bourdichon *et al.*, 2011
Lactobacillus parabuchneri	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus paracasei subsp paracasei	Cheese fermentation, Probiotic cheese, Probiotics, Wine	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
	Meat	Bernardeau *et al.*, 2006;
Lactobacillus parakefiri	Dairy	Bourdichon *et al.*, 2011
Lactobacillus paralimentariums	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus paraplantarum	Ricotta	Bernardeau *et al.*, 2006
	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus pentosus	Meat fermentation and biopreservation of meat	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
	Green table olives	
	Dairy, Fruits, Wine	Bernardeau *et al.*, 2006;
Lactobacillus plantarum subsp. plantarum	Fermentation of vegetables, Malolactic fermentation, Green table olives	Mogensen *et al.*, 2002b; Bourdichon et al., 2011

	Dairy, Meat	
Lactobacillus pobuzihii	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus pontis	Sourdough	Bernardeau *et al.*, 2006
Lactobacillus rapis	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus reuteri	Sourdough	Bernardeau *et al.*, 2006
Lactobacillus rhamnosus	Dairy, Vegetables, Meat	Bourdichon *et al.*, 2011
Lactobacillus rossiae	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus sakei subsp. carnosus	Meat fermentation	Mogensen *et al.*, 2002b
Lactobacillus sakei subsp. sakei	Fermentation of cheese and meat products Beverages	Mogensen *et al.*, 2002b; Bourdichon et al., 2011
Lactobacillus salivarious subsp. salivarius	Cheese fermentation	Mogensen *et al.*, 2002b
Lactobacillus sanfranciscensis	Sourdough	Mogensen *et al.*, 2002b; Bernardeau *et al.*, 2006
Lactobacillus satsumensis	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus secaliphilus	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus senmaizukei	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus siliginis	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus similis	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus spicheri	Sourdough	Bourdichon *et al.*, 2011
Lactobacillus suebicus	Fruits	Bourdichon *et al.*, 2011
Lactobacillus sunkii	Vegetables	Bourdichon *et al.*, 2011
Lactobacillus tucceti	Dairy, Meat	Bourdichon *et al.*, 2011
Lactobacillus vaccinostercus	Fruits, Vegetables, Cocoa	Bourdichon *et al.*, 2011
Lactobacillus versmoldensis	Dry sausages	Bernardeau *et al.*, 2006
Lactobacillus yamanashiensis	Beverages	Bourdichon *et al.*, 2011
Lactococcus		
Lactococcus lactis subsp. cremoris	Dairy starter	Mogensen *et al.*, 2002b
Lactococcus lactis subsp. lactis	Dairy starter[1], Nisin (food additive)[2]	Mogensen *et al.*, 2002b[1]; Rani and Soni, 2007[2]
Lactococcus raffinolactis	Dairy	Bourdichon *et al.*, 2011
Leuconostoc		
Leuconostoc carnosum	Meat, bioprotection	Hansen, 2004 Bourdichon *et al.*, 2011
Leuconostoc citreum	Dairy, Fish	Bourdichon *et al.*, 2011
Leuconostoc fallax	Vegetables	Bourdichon *et al.*, 2011

Leuconostoc holzapfelii	Coffee	Bourdichon *et al*., 2011
Leuconostoc inhae	Vegetables	Bourdichon *et al*., 2011
Leuconostoc kimchii	Vegetables	Bourdichon *et al*., 2011
Leuconostoc lactis	Dairy starter	Mogensen *et al*., 2002b
Leuconostoc mesenteroides subsp. cremoris	Dairy starter	Mogensen *et al*., 2002b
Leuconostoc mesenteroides subsp. dextranicum	Dairy starter	Mogensen *et al*., 2002b
Leuconostoc mesenteroides subsp. mesenteroides	Dairy starter	Mogensen *et al*., 2002b
Leuconostoc palmae	Wine	Bourdichon *et al*., 2011
Leuconostoc pseudomesenteroides	Dairy	Hansen, 2004
Macrococcus		
Macrococcus caseolyticus	Dairy, Meat	Bourdichon *et al*., 2011
Microbacterium		
Microbacterium gubbeenense	Soft cheese ripening	Bockelmann *et al.*, 2005
Micrococcus		
Micrococcus luteus	Cheese ripening	Bourdichon *et al*., 2011
Micrococcus lylae	Meat fermentation	Bourdichon *et al*., 2011
Micrococcus lysodeikticus	Catalase (food additive)	Rani and Soni, 2007
Oenococcus		
Oenococcus oeni	Malolactic fermentation of wine	Mogensen *et al*., 2002b
Pediococcus		
Pediococcus acidilactici	Meat fermentation and biopreservation of meat Cheese starter	Mogensen *et al*., 2002b
Pediococcus damnosus	Meat, bioprotection	Hansen, 2004
Pediococcus parvulus	Wine	Bourdichon *et al*., 2011
Pediococcus pentosaceus	Meat fermentation and biopreservation of meat	Mogensen *et al*., 2002b
Propionibacterium		
Propionibacterium acidipropionici	Meat fermentation and biopreservation of meat	Mogensen *et al*., 2002b
Propionibacterium arabinosum	Cheese fermentation Probiotic cheese Probiotics	Mogensen *et al*., 2002b
Propionibacterium freudenreichii subsp. freudenreichii	Cheese fermentation (Emmental cheese starter)	Mogensen *et al*., 2002b
Propionibacterium freudenreichii subsp. shermanii	Cheese fermentation (Emmental cheese starter)	Mogensen *et al*., 2002b
Propionibacterium jensenii	Dairy	Bourdichon *et al*., 2011
Propionibacterium thoenii	Biopreservation of foods	Mogensen *et al*., 2002b
Staphylococcus		

Staphylococcus carnosus subsp. carnosus	Meat fermentation and biopreservation of meat	Mogensen *et al.*, 2002b
Staphylococcus carnosus subsp. utilis	Meat fermentation	Mogensen *et al.*, 2002b
Staphylococcus cohnii	Dairy, Meat	Bourdichon *et al.*, 2011
Staphylococcus condimenti	Soy	Bourdichon *et al.*, 2011
Staphylococcus equorum subsp. equorum	Dairy, Meat	Bourdichon *et al.*, 2011
Staphylococcus equorum subsp. linens	Dairy	Bourdichon *et al.*, 2011
Staphylococcus fleurettii	Dairy	Bourdichon *et al.*, 2011
Staphylococcus piscifermentans	Fish	Bourdichon *et al.*, 2011
Staphylococcus saprophyticus	Meat	Bourdichon *et al.*, 2011
Staphylococcus sciuri subsp. sciuri	Cheese fermentation	Mogensen *et al.*, 2002b
Staphylococcus succinus subsp. succinus	Meat	Bourdichon *et al.*, 2011
Staphylococcus succinus subsp. casei	Dairy	Bourdichon *et al.*, 2011
Staphylococcus vitulinus	Meat fermentation Dairy	Mogensen *et al.*, 2002b Bourdichon *et al.*, 2011
Staphylococcus warneri	Meat	Bourdichon *et al.*, 2011
Staphylococcus xylosus	Dairy	Bourdichon *et al.*, 2011
Streptococcus		
Streptococcus gallolyticus subsp. macedonicus	Dairy	Bourdichon *et al.*, 2011
Streptococcus salivarius subsp. salivarius	Soy, Vegetables	Bourdichon *et al.*, 2011
Streptococcus salivarius subsp. thermophilus	Dairy	Bourdichon *et al.*, 2011
Streptomyces		
Streptomyces griseus subsp griseus	Meat[1], Cyanocobalamin-B_{12} (food additive)[2]	Bourdichon *et al.*, 2011[1]; Rani and Soni, 2007[2]
Streptomyces natalensis	Natamycin (food additive)	Rani and Soni, 2007
Streptomyces olivaceous	Glucose isomerase (food additive)	Rani and Soni, 2007
Tetragenococcus		
Tetragenococcus halophilus	Soy sauce	Bourdichon *et al.*, 2011
Tetragenococcus koreensis	Vegetables	Bourdichon *et al.*, 2011
Weissella		
Weisella confusa	Meat Sourdough	Bourdichon *et al.*, 2011
Weisella beninensis	Vegetables	Bourdichon *et al.*, 2011
Weisella cibaria	Vegetables	Bourdichon *et al.*, 2011
Weisella fabaria	Cocoa	Bourdichon *et al.*, 2011
Weisella ghanensis	Cocoa	Bourdichon *et al.*, 2011

Weisella hellenica	Meat	Bourdichon *et al*., 2011
Weisella koreensis	Vegetables	Bourdichon *et al*., 2011
Weisella paramesenteroides	Meat	Bourdichon *et al*., 2011
Weisella thailandensis	Fish	Bourdichon *et al*., 2011
Weisella halotolerans	Meat	Hansen, 2004
Zymomonas		
Zymomonas mobilis subsp. mobilis	Beverages	Bourdichon *et al*., 2011
Mycelium fungi / Moulds		
Actinomucor		
Actinomucor elegans	Fresh tofu	Han *et al*., 2003
Aspergillus		
Aspergillus acidus	Tea	Bourdichon et al., 2011, Mogensen et al., 2009
Aspergillus flavus	α-amylases (food additive)	Rani and Soni, 2007
Aspergillus glaucus	Fermentation of soybean wheat curd	Blandino *et al*., 2003
Aspergillus niger	Beverages[1] Industrial production of citric acid[2] Amyloglucosidases, Pectinase, Cellulase, Glucose oxidase, Protease (food additives)[3]	Bourdichon *et al*., 2011[1]; Vandenberghe *et al*., 2000[2]; Rani and Soni, 2007[3]
Aspergillus oryzae	Soy sauce, Beverages[1] α-amylases, Amyloglucosidase, Lipase (food additives)[2]	Bourdichon *et al*., 2011[1]; Rani and Soni, 2007[2]
Aspergillus sojae	Soy sauce	Valyasevi and Rolle, 2002
Fusarium		
Fusarium solani	Cheese production	Mogensen *et al*., 2002b
Fusarium domesticum	Dairy	Bourdichon *et al*., 2011
Fusarium venenatum	Dairy	Bourdichon *et al*., 2011
Lecanicillium		
Lecanicillium lecanii	Dairy	Bourdichon *et al*., 2011
Mucor		
Mucor hiemalis	Fresh tofu	Bourdichon *et al*., 2011 Handbook p.491
Mucor indicus	Rice fermentation	Blandino *et al*., 2003
Mucor mucedo	Dairy	Bourdichon *et al*., 2011
Mucor plumbeus	Dairy	Bourdichon *et al*., 2011

Mucor racemosus	Dairy	Bourdichon *et al*., 2011
Mucor rouxianus	Rice fermentation	Blandino *et al*., 2003
Neurospora		
Neurospora sitophila	Vegetables	Bourdichon *et al*., 2011
Penicillium		
Penicillium camemberti	White mould cheeses (camembert type)	Mogensen *et al*., 2002b
Penicillium caseifulvum	Dairy	Bourdichon *et al*., 2011
Penicillium chrysogenum	Dairy[1], Glucose oxidase (food additive)[2]	Mogensen *et al*., 2002b[1]; Rani and Soni, 2007[2]
Penicillium citrinum	Nuclease (food additive)	Rani and Soni, 2007
Penicillium commune	Dairy	Bourdichon *et al*., 2011
Penicillium nalgiovense	Meat (sausage) fermentation Dairy	Mogensen *et al*., 2002b Bourdichon *et al*., 2011
Penicillium notatum	Glucose oxidases (food additive)	Rani and Soni, 2007
Penicillium roqueforti	Blue mould cheeses	Mogensen *et al*., 2002b
Penicillium solitum	Meat	Bourdichon *et al*., 2011
Rhizopus		
Rhizopus chinensis	Fermentation of rice	Blandino *et al*., 2003
Rhizopus microspores	Vegetables	Bourdichon *et al*., 2011
Rhizopus niveus	Amyloglucosidases (food additive)	Rani and Soni, 2007
Rhizopus oligosporus	Soy	Bourdichon *et al*., 2011
Rhizopus oryzae	Soy	Bourdichon *et al*., 2011
Rhizopus stolonifer	Soy	Bourdichon *et al*., 2011
Scopulariopsis		
Scopulariopsis flava	Dairy	Bourdichon *et al*., 2011
Sporendonema		
Sporendonema casei	Dairy	Bourdichon *et al*., 2011
Yeasts		
Candida		
Candida etchellsii	Dairy, Soy, Vegetables	Bourdichon *et al*., 2011
Candida famata	Fermentation of blue vein cheese and biopreservation of citrus Meat	Mogensen *et al*., 2002b; Hansen, 2004
Candida friedricchi	Kefir fermentation	Mogensen *et al*., 2002b
Candida guilliermondii	Citric acid (food additive)	Rani and Soni, 2007
Candida holmii	Kefir fermentation	

Candida krusei	Kefir fermentation Sour dough fermentation	Mogensen *et al.*, 2002b Bockelmann et al., 2005
Candida lipolytica	Citric acid (food additive)	Rani and Soni, 2007
Candida milleri	Sourdough	Bourdichon *et al*., 2011
Candida oleophila	Wine	Bourdichon *et al*., 2011
Candida pseudotropicalis	Lactase (food additive)	Rani and Soni, 2007
Candida rugosa	Dairy	Bourdichon *et al*., 2011
Candida tropicalis	Vegetables	Bourdichon *et al*., 2011
Candida utilis	Fortification of corn meal by fermentation	Mogensen *et al.*, 2002b
Candida valida	Used for cheese ripening	Mogensen *et al.*, 2002b
Candida versatilis	Dairy, Soy	Bourdichon *et al*., 2011
Candida zemplinina	Wine	Bourdichon *et al*., 2011
Candida zeylanoides	Dairy	Bourdichon *et al*., 2011
Pichia kudriavzevii	Diary, Cocoa	Bourdichon *et al*., 2011
Pichia occidentalis	Dairy, Vegetables	Bourdichon *et al*., 2011
Cyberlindnera		
Cyberlindnera jadinii	Dairy	Bourdichon *et al*., 2011
Cyberlindnera mrakii	Wine	Bourdichon *et al*., 2011
Cystofilobasidium		
Cystofilobasidium infirmominiatum	Dairy	Bourdichon *et al*., 2011
Debaryomyces		
Debaryomyces hansenii	Ripening of smear cheeses Meat	Bockelmann et al., 2005; Bourdichon *et al*., 2011
Dekkera		
Dekkera bruxellensis	Beverages	Bourdichon *et al*., 2011
Hanseniaspora		
Hanseniaspora guilliermondii	Wine	Bourdichon *et al*., 2011
Hanseniaspora osmophila	Wine	Bourdichon *et al*., 2011
Hanseniaspora uvarum	Wine	Bourdichon *et al*., 2011
Kazachstania		
Kazachstania exigua	Dairy, Sourdough	Bourdichon *et al*., 2011
Kazachstania unispora	Dairy	Bourdichon *et al*., 2011
Galactomyces		
Galactomyces candidum	Dairy	Bourdichon *et al*., 2011
Geotrichum		

Geotrichum candidum	Ripening of soft and semisoft cheeses or fermented milks Meat	Mogensen *et al.*, 2002b Bockelmann *et al.*, 2005; Bourdichon *et al*., 2011
Guehomyces		
Guehomyces pullulans	Vegetables	Bourdichon *et al*., 2011
Kluyveromyces		
Kluyveromyces lactis	Cheese ripening[1] Lactase (food additive)[2]	Mogensen *et al.*, 2002b[1], Rani and Soni, 2007[2]
Kluyveromyces marxianus	Fermentation of soy milk Fortification of soft cheese Flavor enhancer	Mogensen *et al.*, 2002b
Lachancea		
Lachancea fermentati	Wine	Bourdichon *et al*., 2011
Lachancea thermotolerans	Wine	Bourdichon *et al*., 2011
Metschnikowia		
Metschnikowia pukcherrima	Wine	Bourdichon *et al*., 2011
Pichia		
Pichia fermentans	Isolated from fermented olives Dairy, Wine	Mogensen *et al.*, 2002b Bourdichon *et al*., 2011
Pichia kluyverii	Wine	Bourdichon *et al*., 2011
Pichia membranifaciens	Dairy	Bourdichon *et al*., 2011
Pichia pijperi	Wine	Bourdichon *et al*., 2011
Saccharomyces		
Saccharomyces bayanus	Kefir fermentation Juice and wine fermentation	Mogensen *et al.*, 2002b
Saccharomyces cerevisiae	Beer[1], Bread[2], Invertase (food additive)[3]	Mogensen *et al.*, 2002b[1]; Caplice and Fitzerald, 1999[2]; Rani and Soni, 2007[3]
Saccharomyces cerevisiae subsp. boulardii	Used as probiotic culture	Mogensen *et al.*, 2002b
Saccharomyces florentius	Kefir fermentation	Mogensen *et al.*, 2002b
Saccharomyces pastorianus	Beer	Hansen, 2004
Saccaharomyces sake	Fermentation of rice	Blandino *et al*., 2003
Saccharomyces unisporus	Kefir fermentation	Mogensen *et al.*, 2002b
Torulopsis candida	Vegetables	Bourdichon *et al*., 2011
Torulopsis holmii	Vegetables	Bourdichon *et al*., 2011
Schizosaccharomyces		
Schizosaccharomyces pombe	Wine	Bourdichon *et al*., 2011

Schwanniomyces		
Schwanniomyces vanrijiae	Wine	Bourdichon *et al*., 2011
Starmerella		
Starmerella bombicola	Wine	Bourdichon *et al*., 2011
Torulaspora		
Torulaspora delbrueckii	Dairy, Wine	Bourdichon *et al*., 2011
Trigonopsis		
Trigonopsis cantarellii	Wine	Bourdichon *et al*., 2011
Wickerhamomyces		
Wickerhamomyces anomalus	Wine	Bourdichon *et al*., 2011
Yarrowia		
Yarrowia lipolytica	Dairy	Bourdichon *et al*., 2011
Zygosaccharomyces		
Zygosaccharomyces rouxii	Soy sauce	Bourdichon *et al*., 2011; Caplice and Fitzerald, 1999
Zygotorulaspora		
Zygotorulaspora florentina	Dairy	Bourdichon *et al*., 2011

TABLE II

Representative traditional fermented legume products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate / raw material	Sensory features and nature	Microorganisms	Country / References
Aakhone	Soybean	Alkaline, sticky, paste	*Bacillus* sp.	India
Bekang	Soybean	Alkaline, sticky, paste	*B. subtilis, B. pumilus, B. lichniformis, B. sphaericus, B. brevis, B. coagulans, B. circulans, Ent. faecium, Ent. hirae, Ent. raffinossus, Ent. duran, Ent. cecorum, Sacch. cerevisiae, Deb. hansenii, P. burtonii*	India / Das and Deka, 2012; Tamang *et al.*, 2012
Bhallae	Black gram	Mild acidic	*B. subtilis, Cand. curvata, Cand. famata, Cand. membraneafaciens, Cand. variovaarai, Cryptococcus humicoius, Deb. hansenii, Geotrichum candidum, Hansenula anomala, Hasenula polymorpha, Kl. marxianus, Lb. fermentum, Leuc. mesenteroides, P. membranaefaciens, Rhiz. marina, Sacch. cerevisiae, Ent. faecalis, Trichosporon beigelii, Trichosporon pullulans, Wingea robertsii*	India / Rani and Soni, 2007
Bikalga	Roselle (*Hibiscus Sabdariffa*)	Condiment	*B. subtilis, B. licheniformis, B. megaterium, B. pumilus*	Burkina Faso / Ouoba *et al.*, 2007
Ce-lew	Soybean, corn flour, rice flour, salt	Soya sauce	*Ped. halopholus, Bacillus* sp., *Asp. oryzae, Asp. flavus*	Thailand
Chee-fan	Soybean whey curd	Cheese-like, solid	*Mucor* sp., *Asp. glaucus*	China / Blandino *et al.*, 2003
Chiang	Soybean	Alkaline, paste	Moulds	China
Cheonggukjang	Soybean	Alkaline, sticky	*B. subtilis, B. amyloliquefaciens, B. licheniformis, B. cereus,Pantoea agglomerans, Pantoega ananatis, Enterococcus* sp., *Pseudomonas* sp., *Rhodococcus* sp.	Korea / Ahn *et al.*, 2006; Hong *et al.*, 2012
Dage	Coconut press cake, *Ragi*		*Rhizopus* sp.,	*Indonesia*
Douchi	Soybean	Alkaline, paste	*B. amyloliquefaciens,B. subtilis,* moulds	China, Taiwan / Peng *et al.*, 2003; Wang *et al.*, 2006
Dawadawa	Locust bean	Alkaline, sticky	*B. pumilus, B. licheniformis, B. subtilis, B. firmus, B. atrophaeus, B.amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus.*	Ghana, Nigeria / Amoa-Awua *et al.*, 2006; Meerak *et al.*, 2008

Dhokla	Bengal gram	Mild acidic, spongy	*Leuc. mesenteroides, Lb. fermenti, Ent. faecalis, Tor. candida, Tor. pullulans*	India / Blandino *et al.*, 2003
Doenjang	Soybean	Alkaline, paste	*B. subtilis, B. licheniformis, B. pumilis, Mu. plumbeus,* Asp. *oryzae, Deb. hansenii, Leuc. mesenteroides, Tor. halophilus, Ent. faecium, Lactobacillus* sp.	Korea / Kim *et al.*, 2009
Dosa	Rice, blackgram Dhal (*Phaselus mango*)	Fermented fan cake	*Leuc. mesenteroides, Lb. delbrueckii, Lb. fermenti, Ent. faecalis, B. amyloliquefaciens, Cand. boidini, Cand. glabrata, Cand. sake, Deb. hansenii, Hansenula polymorpha, Issatchenkia terricola, Rhiz. graminis*	India / Rani and Soni, 2007
Furu	Soybean curd	Mild acidic	*B. pumilus, B. megaterium, B. stearothermophilus, B. firmus, Staph. hominis*	China / Sumino *et al.*, 2003
Hawaijar	Soybean	Alkaline, sticky	*B. subtilis, B. licheniformis, B. cereus, Staph. aureus, Staph. sciuri, Alkaligenes* sp., *Providencia rettger*	India / Das and Deka, 2012
Hishiho-Miso	Soybean, barley or wheat, salt, vegetables, Mizuame, sugar, shoyu	Sweetend Miso	*Asp. oryzae, Ped. halophilus, Sacch. rouxii, Streptococcus* sp.	Japan
Idli	Rice, blackgram Dhal or other dehusked pulses	Germented cereal pudding	*Leuc. mesenteroides, Lb. delbrueckii, Lb. fermenti, Lb. coryniformis, Ped. acidilactis, Ped. cerevisae, Streptococcus* sp., *Ent. faecalis, Lact. lactis, B. amyloliquefaciens, Cand. cacaoi, Cand. fragicola, Cand. glabrata, Cand. kefyr, Cand. pseudotropicalis, Cand. sake, Cand. tropicalis, Deb. hansenii, Deb. tamarii, Issatchenkia terricola, Rhiz. graminis, Sacch. cerevisiae, Tor. candida, Tor. holmii,*	India / Rani and Soni, 2007
Iru	Locust bean	Alkaline, sticky	*B. subtilis, B. pumilus, B. licheniformis, B. megaterium, B. fumus, B. atrophaeus, B.amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus, Staph. saprophyticus*	Nigeria, Benin / Odunfa and Oyewole, 1997; Meerak *et al.*, 2008
Kanjang	Soybean, meju, salt, water	Soya sauce	*Asp. oryzae, B. subtilis, B. pumillus, B. citreus, Sarcina mazima, Sacch. rouxii*	Korea
Kawal	Leaves of legume (*Cassia* sp.)	Alkaline, strong flavored, dried balls	*B. subilis, propionibacterium* sp., *Lb. plantarum, Staph. sciuri,* Yeasts	Sudan / Dirar *et al.*, 2006
Kecap	Soybean, wheat	Liquid	*Rhiz. oligosporus, Rhiz. oryzae, Asp. oryzae, Ped. halophilus, Staphylococcus* sp., *Candida* sp., *Debaromyces* sp., *Sterigmatomyces* sp.	Indonesia
Ketjap	Soybean (black)	Syrup	*Asp. oryzae, Asp. flavus, Rhiz. oligosporus, Rhiz. arrhizus*	Indonesia

Kinda	Locust bean	Alkaline, sticky	*B. pumilus, B. licheniformis, B. subtilis, B. atrophaeus, B.amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus.*	Sierra Leone / Meerak *et al.*, 2008
Kinema	Soybean	Alkaline, sticky	*B. subtilis, B. licheniformis, B. cereus, B. circulans, B. thuringiensis, B.* sp*haericus, Ent. faecium, Cand. parapsilosis, Geotrichum candidum*	India, Nepal, Bhutan / Das and Deka, 2012; Sarkar *et al.*, 2002
Khaman	Bengal gram	Mild acidic, spongy	*Leuc. mesenteroides, Lb. fermentum, Lact. lactis, Ped. acidilactici, Bacillus* sp.	India
Koikuchi Shoyu	Defatted soybean flake, wheat, brine, tane-koji	Soy sauce	*A*sp. *sojae, A*sp. *oryzae, Sacch. rouxii, Tor. versatilis, Tor. echellsii, Ped. halophilus, Sacch. halomembransis, Ent. faecalis, Bacillus.* sp.	Japan
Koji	Soybean		*A*sp. oryzae, *A*sp. *awamori*, *A*sp. *usamii*	China / Raimbault *et al.*, 1985
Maseura	Black gram	Dry, ball-like, brittle	*B. subtilis, B. mycoides, B. pumilus, B. laterosporus, Ped. acidilactici, Ped. pentosaceous, Ent. durans, Lb. fermentum, Lb. salivarius, Sacch. cerevisiae, P. burtonii, Cand. castellii*	Nepal, India / Tamang *et al.*, 2012
Meitauza	Soybean	Liquid	*B. subtilis, A*sp. *oryzae, Rhiz. oligosporus, Mu. meitauza, Actinomucor elegans*	China, Taiwan / Zhu *et al.*, 2008
Meju	Soybean	Alkaline, paste	*A*sp. *oryzae, B. subtilis, B. pumilus, Sarcina maxima, Sacch. rouxii*	Korea
Miso	Soybean	Alkaline, paste	*Ped. acidilactici, Leuc. paramesenteroides, Micrococcus halobius, Zygosaccharomyces rouxii, A*sp. *oryzae*	Japan / Asahara *et al.*, 2006
Miso (Hishiho)	Soyban, barley or wheat, salt, vegetables, Mizuame (dextrose syrup), sugar, Shoyu	Sweet miso	*A*sp. *oryzae, Ped. halophilus, Sacch. rouxii, Streptococcus* sp.	Japan
Miso (Kome Ama)	Rice, soybean, salt, Tane-koji	Sweet rice miso	*A*sp.*oryzae, Streptococcus* sp., *Pediococcus* sp., *Sacch. rouxii*	Japan
Miso (Kome Kara)	Rice, soybean, salt, Tane-koji, salt	Salt rice miso	*A*sp. *oryzae, Sacch. rouxii, Ped. halophilus, Tor. versalis, Tor. echellsii, Bacillus* sp.	Japan
Miso (Mame)	Cereal, soybean, salt		*A*sp. *oryzae, A*sp. *sojae, Ent. faecalis, Tor. versatilis, Bacillus* sp.	Japan
Miso (Mugi)	Barley, soybean, salt, koji	Barley Miso	*A*sp. *oryzae, Sacch. rouxii, Ped. halophilus, Ent. faecalis, Tor. versatilis, Tor. echellsii, Bacillus* sp.	Japan
Moromi	Soybean		*Aspergillus* sp., *Sacch. rouxii*	Japan / FAO, 1998; FAO 1999
Natto	Soybean	Alkaline, sticky	*A*sp. *oryzae, B. subtilis*	Japan

Oncon Hitam	Peanut press cake, tapioca, soybean curd starter	Fermented peanut press cake	*Mucor* sp., *Rhizopus* sp.	Indonesia
Ogiri / Ogili	Melon Seeds, castor oil seeds, pumpkin bean, sesame		*B. subtilis, B. pumilus, B. licheniformis, B. megaterium, B. rimus, Pediococcus* sp., *Staph. saprophyticus, Lb. plantarum*	West, East and Central Africa / Odunfa and Oyewole, 1997
Okpehe	Seeds from *Prosopis africana*	Alkaline, sticky	*B. subtilis, B. amyloliquefaciens, B. cereus, B. licheniformis,*	Nigeria / Oguntoyinbo *et al.*, 2010
Oncom Merah	Peanut press cake, tapioca, bulgur, soybean curd starter		*Neurospora* sp.	Indonesia
Owoh	Cotton seed		*B. subtilis, B. pumilus, B.licheniformis, Staph. saprophyticus*	West Africa / Odunfa and Oyewole, 1997
Papad	Black gram	Circular wafers	*Cand. krusei, Deb. hansenii, Lb. fermentum, Leuc. mesenteroides, P. membranaefaciens, Sacch. cerevisiae, Ent. faecalis, Trichosporon beigelii*	India, Nepal / Rani and Soni, 2007
Pepok	Soybean	Alkaline, sticky	*Bacillus* sp.	Myanmar
Peruyaan	Soybean	Alkaline, sticky	*B. subtilis, Baciilus* sp., LAB	India / Das and Deka, 2012
Sieng	Soybean	Alkaline, sticky	*Bacillus* sp.	Cambodia, Laos
Soumbala	Locust bean	Alkaline, sticky	*B. pumilus, B. atrophaeus, B.amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus. B. subtilis, B. thuringiensis, B. licheniformis, B. cereus, B. badius, B. firmus, B. megaterium, B. mycoides, B.* sp*haericus, Peanibacillus. alvei, Peanibacillus larvae, Brevibacillus laterosporus*	Burkina Faso / Sarkar *et al.*, 2002; Meerak *et al.*, 2008
Soy sauce	Soybean	Alkaline, liquid	*Asp. oryzae, Asp. niger, Sacch. rouxii, Ped. acidilactis, Ped. cerevisae, Ped. halophilus, Lb. delbrueckii*	Worldwide / Rani and Soni, 2007
Sufu	Soybean curd	Mild-acidic, soft	*Actinomucor elenans, Mu. silvatixus, Mu. corticolus, Mu. hiemalis, Mu. praini, Mu. racemosus, Mu. subtilissimus, Rhiz. chinensis*	China, Taiwan / Rani and Soni, 2007
Tai-Tio	Soybeans and roasted wheat, glutinous rice		*Asp. oryzae*	East India
Tamari Shoyu	Defatted soybean, salt, water, wheat	Soybean sich Shoyu	*Asp. sojae, Asp. oryzae, Sacch. rouxii, Tor. versaltilis, Tor. echellsii, Ped. halophilus, Ent. faecalis, Bacillus* sp.	Japan
Tauco	Soybean	Alkaline, paste	*Rhiz. oryzae, Rhiz. ologosporus, Asp. oryzae, Lb. delbrueckii, Zygosaccharomyces soyae*	Indonesia / Rani and Soni, 2007

Tao-si	Soybean, salt, rice bran, wheat flour	Fermented soybean curd	*Asp. oryzae*	Philippines / Blandino *et al.*, 2003
Tempe	Soybean	Alkaline, solid	*Asp. niger, Rhiz. oligisporus, Rhiz. arrhizus, Rhiz. oryzae, Rhiz. stolonifer, Klebsiella pneumoniae*	Indonesia (Origin), The Netherlands, Japan, USA / Rani and Soni, 2007
Tempe Benguk	Velvet bean seeds, *Ragi* Tempe		*Rhizopus* sp., *Rhiz. oligosporus, Rhiz. arrhizus*	Indonesia
Tempe Gembus	Solid residue of soybean curd, tapioca, *Ragi* tempe		*Rhizopus* sp. *Rhiz. oryzae, Rhiz. oligosporus*	Indonesia
Tempe Kecipir	Winged bean seed, *Ragi*, old Tempe		*Rhiz. oryzae, Rhiz. arrhizus, Rhiz. oligosporus, Rhiz.achlamydosporus*	Indonesia
Tempe Kedelai	Soybean, tapioca flour, maize, young papaya, cassava, coconut press cake, starter		*Rhizopus* sp., *Rhiz. oryzae, Rhiz. oligosporus*	Indonesia
Tempe Koro Pedang	Jack bean seed(*Canavalia ensiformis*), *Ragi*, old Tempe		*Rhiz. oryzae, Rhiz. arrhizus, Rhiz. achlamydosporus*	Indonesia
Tempe Lamtoro	Wied Tamarind (*Leucaena Leucocephala*)		*Rhizopus* sp., *Rhiz. oryzae*	Indonesia
Thua nao	Soybean	Alkaline, paste, dry	*B. subtilis, B. pumilus, Lactobacillus* sp.	Thailand
Tofu (stinky tofu)	Soybean	Alkaline, liquid	*Bacillus sp., Ent. hermanniensis, Enterococcus sp., Lb. agilis, Lb. brevis, Lb. buchneri, Lb. crispatus, Lb. curvatus, Lb. delbrueckii, Lb. farciminis, Lb. fermentum, Lb. pantheris, Lb. salivarius, Lb. vaccinostercus, Lact. lactis, Lactococcus sp., Leuc. camosum, Leuc. citreum, Leuc. fallax, Leuc. lactis, Leuc. mesenteroides, Leuc. pseudomesenteroides, Ped. acidilactici, Strep. bovis, Strep. macedonicus, W. cibaria, W. confusa, W. paramesenteroides, W. soli*	China, Japan / Chao *et al.*, 2008
Toyo	Soybean, salt, brown sugar, wheat starter	Cowpea sauce	*Asp. oryzae, Hansenula anomala, Hansenula subpelliculosa, Lb. delbrueckii*	Philippines
Tungrymbai	Soybean	Alkaline, sticky	*B. subtilis, B. pumilus, B. licheniformis, Ent. faecium,Ent. hirae, Ent. raffinossus, Ent. durans, Ent. cecorum,Lb. brevis, Cand. parapsilosis, Sacch. bayanus,Sacch. cerevisiae, Deb. hansenii, P. burtonii, Sm. fibuligera, Geotrichum candidum*	India / Das and Deka, 2012; Tamang *et al.*, 2012
Tuong	Rice, maize, salt		*Asp. oryzae, Sacch. rouxii, Ped. halophilus*	Vietnam

Ugba	African oil bean (*Pentaclethra macrophylla*)	Alkaline, flat, glossy, brown in color	*B. subtilis, B. pumilus, B. licheniformis, Staph. saprophyticus*	Nigeria / Odunfa and Oyewole, 1997
Usukuchi Shoyu	Soybean, wheat, Tane-Koji, Amasake	Soy sauce	*Asp. oryzae, Sacch. rouxii, tor. versatilis, Tor. echellsii, Ped. halophilus, Sacch. halomembransis, Ent. faecalis, Bacillus* sp.	Japan
Vadai	Black gram	Paste	*Pediococcus* sp., *Streptococcus* sp., *Leuconostoc* sp.	India / Blandino *et al.*, 2003
Wari	Black gram	Ball-like, brittle	*B. subtilis, Cand. curvata, Cand. famata, Cand. krusei, Cand. parapsilosis, Cand. vartiovaarai, Cryptococcus humicolus, Deb. hansenii, Deb. tamarii, Geotrichum candidum, Hansenula anomala, Kl. marxianus, Sacch. cerevisiae, Rhiz. lactosa, Ent. faecalis, Wingea robetsii, Trichosporon beigelii*	India / Rani and Soni, 2007

TABLE III

Representative traditional fermented cereal products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Raw material / Substrate	Sensory property and nature	Microorganisms	Country / References
Abreh	Sorghum	Solid state and submerged	*Lb. plantarum*	Sudan / Odunfa and Oyewole, 1997
Aliha	Maize, sorghum	Non-alcoholic beverage	LAB	Ghana, Togo, Benin
Ambali	Millet, rice	Shallow-fried, staple	LAB	India
Ang-kak	Red rice	Colourant	*Monascus purpureus*	China/ Blandino *et al.*, 2003
Banku	Maize and cassava	Staple food	*Lactobacillus* sp., yeasts	Ghana
Bahtura	Wheat flour	Deep-fried bread	LAB, yeasts	India
Boza	cereals	Sour refreshing liquid	*Lactobacillus* sp., *Lactococcus* sp., *Pediococcus* sp., *Leuconostoc* sp., *Sacch. cerevisiae*	Bulgaria, Balkan / Botes *et al.*, 2007; Blandino *et al.*, 2003
Brem	Glutinous rice		*Sacch. cerevisiae, Cand. glabrata, P. anomala, Issatchenkia arientalis, Lactobacillus* sp., *Acetobacter* sp.	Indonesia / Sujaya *et al.*, 2004
Burukutu	Sorghum and cassava	Creamy, liquid	*Sacch. cerevisiae, Sacch. chavelieri, Leuc. mesenteroides, Candida* sp., *Acetobacter* sp.	Nigeria / Odunfa and Oyewole, 1997
Busa	Maize, Sorghum, Millet	Submerged	*Sacch. cerevisiae, Schizosacchromyces pombe, Lb. plantarum ,Lb. helveticus, Lb. salivarius, Lb. casei, Lb. brevis, Lb. buchneri, Leuc. mesenteroides, Pen. damnosus*	East Africa, Kenya / Odunfa and Oyewole, 1997; Blandino *et al.*, 2003
Ben-saalga	Pearl millet	Weaning food	*Lactobacillus* sp., *Pediococcus* sp., *Leuconostoc* sp., *Weissela* sp., yeasts	Burkina Faso, Ghana / Humblot and Guyot, 2009
Chilra	Wheat, barley, buckwheat	Staple	LAB, *Sacch. cerevisiae*	India
Dégué	Millet	Condient	*Lb. gasseri, Lb. fermentum, Lb. brevis, Lb. casei, Enterococcus* sp.	Burkina Faso / Abriouel *et al.*, 2006
Dhokla	Bengal gram and rice or wheat		*Leuc. mesenteroides* *Ent. faecalis, Trichosporon pullulans*	India / FAO, 1999
Dosa	Rice and black gram	Thin, crisp pancake, Shallow-fried, staple	*Leuc. mesenteroides, Ent. faecalis, Tor. candida, Trichosporon pullulans*	India, Sri Lanka, Malaysia, Singapore / FAO, 1999

Enjera	Tef flour, wheat	Acidic, sourdough, leavened, Pancake-like bread, staple	*Lb. pontis, Lb. plantarum, Leuc. mesenteroides, Ped. cerevisiae, Sacch. cerevisiae, Cand. glabrata*	Ethiopia / Lee, 1997
Gluten-free sourdoughs	buckwheat and/or teff flours	Acidic, sourdough,	*Ped. pentosaceus, Leuc. holzapfelii, Lb. gallinarum, Lb. vaginalis, Lb. sakei, Lb. graminis, W. cibaria*	Experimental / Moroni *et al.*, 2011
Hopper	Rice, coconut water	Steak-baked, Pancake, staple	*Sacch. cerevisiae*, LAB	Sri Lanka
Hussuwa	Sorghum	Cooked dough	*Lb. fermentum, Ped. acidilactici, Ped. pentosaceus*, Yeast	Sudan / Yousif *et al.*, 2010
Hulumur	Sorghum, rice, millet	Non-alcoholic drink	*Leuc. mesenteroides, Lb. plantarum, Lactobacillus* sp.	Sudan, Turkey
Idli	Rice and black gram	Mild-acidic, soft, moist, spongy	*Leuc. mesenteroides, Ent. faecalis, Tor. candida, Trichosporon pullulans*	India, Sri Lanka, Malaysia, Singapore / FAO, 1999
Jalebi	Wheat flour	Crispy sweet, dounot-like	*Sacch. bayanus*	India, Nepal, Pakistan / FAO, 1999
Kenkey	Maize	Acidic, solid, Steamed dumpling, staple, Aflata	*Lb. plantarum, Lb. brevis, Ent. cloacae, Acinetobacter* sp., *Sacch. cerevisiae, Cand. mycoderma*	Ghana /Odunfa and Oyewole, 1997; Oguntoyinbo *et al*., 2011
Khanom-jeen	Rice	Noodle, Staple	*Lactobacillus* sp., *Streptococcus* sp., *Rhizopus* sp., *Mucor* sp.	Thailand / Blandino *et al*., 2003
Khamak (Kao-mak)	Glutinous rice, Look-pang (starter)		*Rhizopus* sp., *Mucor* sp., *Penicillum* sp., *Aspergillus* sp., *Endomycopsis* sp., *Hansenula* sp., *Saccharomyces* sp.	Thailand
Kichudok	Rice	Steamed cake, Side dish	*Leuc. mesenteroides, Ent. faecalis, Saccharomyces* sp.	Korea / Von Mollendorff and Wilhelm, 2008
Kisra	Sorghum	Thin pancake bread, Staple	*Ped. pentosaceus, Lb. confusus, Lb. brevis, Lactobacillus* sp., *Erwinia ananas, Klebsiella pneumoniae, Ent. cloacae, Cand. intermedia, Deb. hansenii, Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp.	Sudan / Mohammed *et al.*, 1991
Kishk	Wheat, milk	Refreshing beverage	*Lb. plantarum, Lb. brevis, Lb. casei, B. subtilis*, Yeasts	Egypt / Blandino *et al*., 2003
Koko	Maize	Prorridge	*Ent. clocae, Acinetobacter* sp., *Lb. plantarum, Lb. brevis, Sacch. cerevisiae, Cand. mycoderma*	Ghana / Blandino *et al*., 2003
Kunu-zaki	white maize, yellow maize, red sorghum	Mild, sour liquid / Porridge, staple	*W. confusa, Strep. lutetiensis, Strep. gallolyticus subsp. macedonicus,*	West Africa, Nigeria
Lao-chao	Rice	Paste, soft, juicy, glurinous desert	*Rhiz. oryzae, Rhiz. chinensis, Chlamydomucor oryzae, Sacchromycopsis* sp.	China / Blandino *et al*., 2003

Maheu	Maize, sorghum, millet	Refreshing beverage	*Lb. delbrueckii*	South Africa / Steinkraus, 2004
Mahewu	Maize	Refreshing beverage	*Lb. delbruchi, Lact. lactis*	South Africa / Blandino *et al.*, 2003
Mawè	Maize	Intermediate product used to prepare beverages, porridges	*Lb. fermentum, Lb. reuteri, Lb. brevis, Lb. confusus, Lb. curvatus, Lb. buchneri, Lb. salivarius, Lact. lactis, Ped. pentosaceus, Ped. acidilactici, Leuc. mesenteroides*	Benin, Togo / Hounhouigan *et al.*, 1993
Masvusvu	Maize	Refreshing beverage	LAB	Zimbabwe
Marchu	Wheat flour	Baked bread	Unknown	India, Pakistan
Mbege	Maize, sorghum, millet	Submerged	*Sacch. cerevisiae, Schizosaccharomyces pombe, Lb. plantarum, Leuc. mesenteroides*	Tanzania / Odunfa and Oyewole, 1997
Me	Rice	Acidic, sour, Condiment	LAB	Vietnam
Minchin	Wheat gluten	Solid, Condiment	*Paceilomyces* sp., *Aspergilus* sp., *Cladosporium* sp., *Fusarium* sp., *Syncephalastum* sp., *Penicillium* sp., *Trichothecium* sp.	China / Blandino *et al.*, 2003
Mungbean starch	Mungbean	Fermented noodle	*Leuc. mesenteroides*	Thailand
Naan	Wheat flour	Leaved bread, baked	*Sacch. cerevisiae*, LAB	India, Pakistan, Afghanistan
Ogi	Maize, sorghum, millet	Mild-acidic, viscous, Porridge, staple	*Lb. plantarum, Lb. pantheris, Lb. vaccinostercus, Corynebacterium* sp., *Aerobacter sp., Cand. mycoderma, Sacch. cerevisiae, Rhodotorula* sp., *Cephalosporium* sp., *Fusarium* sp., Aspergillus sp., *Penicillium sp.*	Nigeria / Odunfa and Oyewole, 1997
Perkarnaya	Rye	Acidic, aerated bread	Yeasts, LAB	Russia
Pito	Maize, sorghum, millet	Submerged	*Geotrichum candidum, Lactobacillus* sp.,*Candida* sp.	West Africa / Odunfa and Oyewole, 1997
Pizza dough	Wheat	Leaved dough	Baker's yeast	World-wide
Poto poto (Gruel)	Maize	Slurry	*Lb. gasseri, Lb. plantarum / paraplantarum, Lb. acidophilus, Lb. delbrueckii, Lb. reuteri, Lb. casei, Bacillus* sp., *Enterococcus* sp., Yeasts	Congo / Abriouel *et al.*, 2006
Pozol	Maize	Mild-acidic, thick viscous, Porridge, staple	*Lactobacillus* sp., *Leuconostoc* sp., *Candida* sp., *Enterobacteriaceae, B. cereus, Paracolobactrum aerogenoides, Agrobacterium azotophilum, Alkaligenes pozolis, E. coli* var. *napolitana, Pseudomonas mexicana, Klebsiella pneumoniae, Saccharomyces* sp., *moulds*	Mexico / FAO, 1998

Puda/Pudla	Maize, Bengal gram	Solid food, pancake	LAB, yeasts	India
Pumpernickel	Rye	Acidic, full-grain, aerated bread; long shelf-life	Yeasts, LAB, as for rye sourdough	Switzerland, Germany
Puto	Rice	Steamed cake, Breakfast	*Leuc. mesenteroides, Ent. faecalis, Ped. cerevisiae*, yeasts	Philippines
Rabadi	Buffalo or cow milk and cereals, pulses	Mild-acidic, thick slurry-like product	LAB, yeasts	India, Pakistan
Sourdough bread	Rye	Sandwich, bread	*Lb. pontis* and *Lb. panis*, *Lb. amylovorus, Lb. acidophilus, Lb. crispatus, Lb. delbrueckii, Lb. fermentum, Lb. reuteri, Sacch. cerevisiae, Issatchenkia orientalis*	Germany, Northern Europe / Gänzle, 2006
San Francisco	(Rye), mainly wheat	Mild-acidic, leavened bread	*Lb. sanfrancisensis*, *Lb. alimentarius, Lb. brevis, Lb. fructivorans, Lb. paralimentarius, Lb. pentosus, Lb. plantarum, Lb. pontis, Lb. spicheri, Leuc. mesenteroides, W. confusa*	USA / Gänzle, 2006
Seera	Wheat grains	Dried , Sweet dish	Unknown	India, Pakistan
Selroti	Rice-wheat flour-milk	Pretzel-like, Deep fried bread, staple	*Leuc. mesenteroides*, *Ent. faecium, Ped. pentosaceus* and *Lb. curvatus, Sacch. cerevisiae*, *Sacch. kluyveri, Deb. hansenii, P. burtonii, Zygosaccharomyces rouxii*	India, Nepal, Bhutan / Yonzan & Tamang, 2010
Siddu	Wheat flour, opium seeds, walnut	Steamed bread, oval-shaped;	*Sacch. cerevisiae*, *Cand. valida*	India
Shamsy bread	Wheat flour	Spongy bread	Yeasts	Egypt
Sourdough bread	Rye, wheat	Mild-acidic, leavened bread	*Lb. sanfranciscensis, Lb. alimentarius, Lb. buchneri, Lb. casei, Lb. delbrueckii, Lb. fructivorans, Lb. plantarum, Lb. reuteri, Lb. johnsonii, Cand.* humili, Issatchenkia orientalis	America, Europe, Australia
Tapai Pulut	Glutinous rice, *Ragi*		*Chlamydomucor* sp., *Endomycopsis* sp., *Hansenula* sp.	Malaysia
Tape Ketan	Glutinous rice, *Ragi*		*Thizopus* sp., *Chlamydomucor* sp., *Candida* sp., *Endomycopsis* sp., *Saccharomyces* sp.	Indonesia
Tepache	Maize, pineapple, apple or orange		*B. subtilis*, *B. graveolus* and the yeasts, *Tor. insconspicna*, *Sacch. cerevisiae* and *Cand. queretana*	Mexico / FAO, 1998
Togwa	cassava, maize, sorghum, millet	fermented gruel or beverage	*Lb. brevis, Lb. cellobiosus, Lb. fermentum, Lb. plantarum and Ped. pentosaceus, Cand. pelliculosa, Cand. tropicalis, Issatchenkia orientalis, Sacch. cerevisiae*	Tanzania / Mugula *et al.*, 2003
Trahana	Sheep milk, wheat	Mild-acidic, sweet-sour, Soup or biscuit	*Lb. bulgaricus, Strep. thermophilus*, yeasts	Cyprus, Greece, Turkey / Karagozlu *et al.*, 2008

Taotjo	Wheat, rice, soybeans	Semi-solid food,(Condiment	*Asp. oryzae*	East Indies / Blandino *et al.*, 2003
Uji	Maize, sorghum, millet, cassava flour	Acidic, sour, Porridge, staple	*Leuc. mesenteroides, Lb. plantarum*	Kenya, Uganda, Tanzania / Odunfa and Oyewole, 1997

TABLE IV

Representative traditional fermented vegetable products*

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate	Sensory property and nature	Microorganisms	Country / reference
Anishi	Taro leaves		LAB	India
Bastanga	Bamboo shoot		LAB	India
Burong mustala	Mustard		*Lb. brevis, Ped. cerevisiae*	Philippines / Rhee *et al.*, 2011
Cucumbers (pickles)	Cucumbers		*Leuc. mesenteroides, Ped. pentosaceus; Lb. brevis, Lb. plantarum*	Europe, USA, Canada
Dhamuoi	Cabbage		*Leuc.* mesenteroides, *Lb. plantarum, Lb. pentoaceticus, Lb. brevis, B. brassicae*	Vietnam
Dakguadong	Mustard leaf	salad	*Lb. plantarum*	Thailand / Rhee *et al.*, 2011
Ekung	Bamboo shoot		*Lb. plantarum, Lb. brevis, Lb. casei, Tor. halophilus*	India / Das and Deka, 2012
Eup	Bamboo shoot		*Lb. plantarum, Lb. fermentum, Lb. brevis, Lb. curvatus, Ped. pentosaceus, Leuc. mesenteroides, Leuc. fallax, Leuc. lactis, Leuc. citreum, Ent. durans*	India / Tamang *et al.*, 2008; 2012
Fu-tsai	Mustard		*Ent. faecalis, Lb. alimentarius, Lb. brevis, Lb. coryniformis, Lb. farciminis, Lb. plantarum, Lb. versmoldensis, Leuc. citreum, Leuc. mesenteroides, Leuc. pseudomesenteroides, Ped. pentosaceus, W. cibaria, W. paramesenteroides*	Taiwan / Chao *et al.*, 2008
Goyang	Wild vegetable		*Lb. plantarum, L. brevis, Lact. lactis, Ent. faecium, Ped. pentosaceus, Candida* sp.	India, Nepal / Das and Deka, 2012; Tamang *et al.*, 2012
Gundruk	Leafy vegetable		*Lb. fermentum, Lb. brevis, Lb. plantarum,Lb. casei, Ped. pentosaceus, Ped. acidilactici, Leuc. fallax, Streptococcus* sp.,	India, Nepal, Bhutan / Das and Deka, 2012; Tamang *et al.*, 2012
Hirring	Bamboo shoot tips		*Lb. brevis, Lb. plantarum, Lb. curvatus, Ped. pentosaceus, Leuc. mesenteroides, Leuc. fallax, Leuc. lactis, Leuc. citreum, Ent. durans, Lact. lactis*	India / Das and Deka, 2012; Tamang *et al.*, 2008
Hom-dong	Red onion	Fermented red onion	*Leuc. mesenteroides, Ped. cerevisiae,*	Thailand

			Lb. plantarum, Lb. fermentum, Lb. buchneri	
Hum-choy	Gai-choy	Chinese sauerkraut	*Pediococcus* sp., *Streptococcus* sp.,	Chinese
Inziang-sang	Mustard leaves		Homofermentative and hetero-fermentative lactobacilli *Lb. plantarum, Lb. brevis, Ped. acidilactici*	India / Das and Deka, 2012; Tamang *et al.*, 2012
Jeruk	Fruits and vegetables		LAB	Malaysia
Khalpi	Cucumber		*Lb. brevis, Lb. plantarum, Ped. pentosaceus, Ped. acidilactici; Leuc. fallax*	India, Nepal / Das and Deka, 2012
Kimchi (beachoo)	Cabbage, green onion, hot pepper, ginger		*Leuc. mesenteroides, Leuc. citreum, Leuc. gasicomitatum, Leuc. kimchii, Leuc. inhae, W. koreensis, W. cibaria, Lb. plantarum, Lb. sakei, Lb. delbrueckii, Lb. buchneri, Lb. brevis, Lb. fermentum, Ped. acidilactici, Ped. pentosaceus*	Korea
Kimchi (Dongchimi)	Radish, salt, water		*Leuc. mesenteroides, Lb. plantarum, Lb. brevis, Ped. cerevisiae,*	Korea
Kimchi (Kakdugi)	Radish, salt, garlic, green onion, hot pepper, ginger		*Leuc. mesenteroides, , Lb. plantarum, Lb. brevis, Ped. cerevisiae,*	Korea
Lung-siej	Bamboo shoot		*Lb. brevis, Lb. plantarum, Lb. curvatus, Ped. pentosaceus, Leuc. mesenteroides, Leuc. fallax, Leuc. lactis, Leuc. citreum, Ent. durans*	India / Tamang *et al.*, 2008
Naw-mai-dong	Bamboo shoots		*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. brevis, Lb. fermentum, Lb. buchneri*	Thailand
Mesu	Bamboo shoot		*Lb. plantarum, Lb. brevis, Lb. curvatus, Leu, citreum, Ped. pentosaceus*	India, Nepal, Bhutan / Das and Deka, 2012; Tamang *et al.*, 2012
Oiji	Cucumber, salt, water	Fermented cucumber	*Leuc. mesenteroides, Lb. brevis, Lb. plantarum, Ped. cerevisiae*	Korea
Olives (fermented)	Olive		*Leuc. mesenteroides, Ped. pentosaceus; Lb. plantarum*	USA, Spain, Portugal, Peru, Chile
Pak-gard-dong	Leafy vegetable, salt, boiled rice		*Lb. plantarum, Lb. brevis, Ped. cerevisiae*	Thailand
Pak-sian-dong	Leaves of *Gynandropis pentaphylla*		*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. germentum, Lb. buchneri*	Thailand
Poi	Taro corms		LAB, yeasts	Hawaii
Sauerkraut	Cabbage		*Leuc. mesenteroides, Ped. pentosaceus; Lb. brevis, Lb. plantarum, Lb. sakei*	Europe, USA, Canada, Australia

Sayur asin	Mustard leaves, cabbage, salt, coconut weater		*Leuc. mesenteroides, Lb. plantarum, Lb. brevis*	Indonesia
Sinnamani	Radish		LAB	Nepal
Soibum	Bamboo shoot		*Lb. plantarum, Lb. brevis, Lb. coryniformis, Lb. delbrueckii, Leuc. fallax, Leuc. Lact. lactis, Leuc. mesenteroides, Ent. durans, Strep. lactis, B. subtilis, B. lichniformis, B. coagulans, Saccharomyces* sp., *Torulopsis* sp.	India / Das and Deka, 2012; Tamang *et al.*, 2012
Soidon	Bamboo shoot tips		*Lb. brevis, Leuc. fallax, Lact. lactis*	India / Das and Deka, 2012; Tamang *et al.*, 2012
Soijim	Bamboo shoot		*Lb. brevis, Lb. plantarum, Lb. curvatus, Ped. pentosaceus, Leuc. mesenteroides, Leuc. fallax, Leuc. lactis, Leuc. citreum, Ent. durans*	India / Tamang *et al.*, 2008
Sinki	Radish tap-root		*Lb. brevis, Lb. plantarum, Lb. fermentum,Lb. casei, Ped. pentosaceus, Ped. acidilactici, Leuc. fallax*	India, Nepal, Bhutan / Das and Deka, 2012; Tamang *et al.*, 2012
Suan-cai	Vegetables		Ped. pentosaceus, Tetragenococcus halophilus	China / Chen *et al.*, 2006
Suan-tsai	Mustard		*Ent. faecalis, Lb. alimentarius, Lb. brevis, Lb. coryniformis, Lb. farciminis, Lb. plantarum, Lb. versmoldensis, Leuc. citreum, Leuc. mesenteroides, Leuc. pseudomesenteroides, Ped. pentosaceus, W. cibaria, W. paramesenteroides*	Taiwan
Sunki	Turnip		*Lb. plantarum, Lb. fermentum, Lb. delbrueckii, Lb. parabuchneri*	Japan / Endo *et al.*, 2008
Takuanzuke	Japanese radish, salt, sugar, Shochu (Japanese spirit)	Pickle radish	*Lb. plantarum, Lb. brevis, Leuc. mesenteroides, Streptococcus* sp., *Pediococcus* sp., yeasts	Japan
Takanazuke	Broad leaved mustard, red pepper, salt, turmeric	Vegetable pickle Takuanzuke	*Ped. halophilus, Lb. plantarum, Lb. brevis*	Japan
Tuaithur	Bamboo shoot		*Lb. plantarum, Lb. brevis, Ped.pentosaceou, Lact. lactis, B. circulans, B. firmus, B sphaericus, B. subtilis*	India / Tamang *et al.*, 2012

***** Vegetable fermentations are typically dominated by LAB, while yeasts, when present, generally are undesired contaminants. Aerobic conditions may particularly promote their growth, resulting in spoilage.

TABLE V

Representative traditional fermented vinegar products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate	Sensory property and nature	Microorganisms	Country / references
Cuka Aren	Sap from flower stalk of Aren	Vinegar	*Acetobacter* sp.	Indonesia
Cuka Nipah	Sap from inflorescence stalk of Nipa fruiticans	Vinegar	*Acetobacter* sp.	Malaysia
Sirca	Gur of molasses or grains	Vinegar	*Sacch. cerevisiae, Acetobacter* sp.	Pakistan
Sirka	Fruit juices or sugar cane juices	Vinegar	*Acetobacter* sp.	Bangladesh
Suka	Coconut water or fruits or sugar or palm sap or rice washings	Vinegar	*Lb. fermentum, Lb. plantarum, Lb. panis, Lb. pontis, W. cibaria, Acetobacter pomoum, Actobacter ghanensis, Acetobacter orientalis, Acetobacter pasteurianus*	Philippines / Dalmacio *et al.*, 2011
Natade coco	Coconut water, coconut skim milk	Film	*Ac. acetic, Ac. xylinum, Leuc. mesenteroides*	Philippines
Nata de pina	Pineapple	Film	*Ac. acetic, Ac. xylinum, Leuc. mesenteroides*	Philippines

TABLE VI

Representative traditional fermented alcohol products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate	Sensory property and nature	Microorganisms	Country / references
Bhaati Jaanr	Glutinous rice	Fermented rice beverage	*Mu. circinelloides, Rhiz. chinensis, Sm. fibuligera, P. anomala, Sacch. cerevisiae, Cand. glabrata, Ped. pentosaceus, Lb. bifermentans*	India / Tamang *et al.*, 2012
Brem Bali	Glutinous rice, Ragi	Brem wine	*Mu. indicus, Cand. parapsilosis*	Indonesia
Bupju	Rice, glutinous rice, water, starter (Nuruk)	Alcoholic beverage	*Saccharomyces* sp.	Korea
Ciu	Molasses, water, Ragi	Alcoholic liquor		Indonesia
Kodo Ko Jaanr	millet	Alcoholic liquor	*Mu. circinelloides, Rhiz. Chinensis, Sm. fibuligera, P. anomala, Sacch. cerevisiae, Cand. glabrata, Ped. pentosaceus, Lb. bifermentans*	India / Tamang *et al.*, 2012
Sake	Polished rice, glucose	Rice wine	*Asp. oryzae, Sacch. cerevisiae, Lb. sakei, Leuc. mesenteroides*	Japan
Fuit wine	Various fresh fruit, sugar, starter	Fruit wine	*Yeasts*	Philippines
Koha	Kiwifruits, sugar	Kiwifruit wine	*Sacch. cerevisiae*	New Zealand
Lambanog	Coconut sap	Coconut toddy	Yeasts	Philippines
Mirin	Polished rice	Mirin	*Asp. oryzae*	Japan
Roselle wine	Roselle fruit, water, sugar	Roselle wine	*Sacch. ellipsoideus var. Montrachet*	Philippine
Shochu	Polished rice, sweet potato, barley, millet, corn	Liquor	*Asp. awamorii, Asp. kawachii, Sacch. cerevisiae*	Japan
Takju	Rice or barley, wheat flour, sweet potato, starter (Nuruk)	Alcohol beverage	*Sacch. cerevisiae, Hansenula anomala, Bacillus sp., Lactobacillus* sp.	Korea
Toddy and Arrakku	Palm sap, sugar	Palm wine	*Sacch. cerevisiae*	Sri Lanka
Turak (Arak)	Aren, coconut, parl sap	Palm wine		Indonesia
Zutho	Rice	Ethnic alcoholic beverage	*Sacch. cerevisiae*	India / Tamang *et al.*, 2012
Seketch	Maize	Alcoholic beverage	*Sacch. cerevisiae, Sacch. chevalieri, Sacch. elegans, Lb. plantarum, Lact. lactis, B. subtilis, Asp. niger, Asp. flavus, Mu. rouxii*	Nigeria / Blandino *et al.*, 2003

TABLE VII

Representative traditional fermented fish products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate	Sensory property and nature	Microorganisms	Country / references
Bagoong Alamang (Bagoong Isda, Bagoong)	Fish/shrimp, salt	Fish/shrimp paste	*Bacillus* sp., *Pediococcus* sp.	Philippines
Balao-balao (Burong Hipon Tagbilao)	Shrimp, rice, salt.	Fermented rice shrimp	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. brevis, Ent. faecalis*	Philippines
Balao-balao (Burong Hipon Tagbieao)	Shrimp, rice, salt	Fermented fish-rice	*Leuc. mesenteroides, Ped. cerevisiae*	Philippines
Belacan (Blacan)	Shrimp, salt	Shrimp paste		Malaysia
Burong-isda	Fresh fish, rice, salt	Fermented fish-rice	*Lb. brevis, Streptococcus* sp.	Philippines
Burong Bangus	Milkfish, rice, salt, vinegar	Fermented milkfish	*Leuc. mesenteroides, Lb. plantarum, W. confusus*	Philippines
Burong Isda	Fish, rice, salt	Fermented fish	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Strep. faecalis, Micrococcus* sp.	Philippines
Budu	Marine fishes, salt, sugar	Muslim sause, fish sause	*Ped. halophilus, Staph. aureus, Staph. epidermidis, B. subtilis, B. laterosporus, Proteus* sp., *Micrococcus* sp., *Sarcina* sp., *Corynebacterium* sp.	Thailand
Gnuchi	Fish (*Schizothorax richardsonii*), salt, tumeric powder	Eat as curry	*Lb. plantarum, Lact. lactis, Leuc. mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, cand. bombicola, Saccharomycopsis* sp.	India / Tamang et al., 2012
Hentak	Finger sized fish (*Esomus danricus*)	Condiment	*Lact. lactis, Lb. plantarum, Lb. fructosus, Lb. amylophilus, Lb. coryniformis, Ent. faecium, B. subtilis, B. pumilus, Micrococcus* sp., *Candida* sp., *Saccharomycopsis* sp.	India / Tamang et al., 2012
Hoi-malaeng pu-dong	Mussel (*Mytilus smaragdinus*), salt	Fermented Mussel	*Ped. halophilus, Staphylococcus* sp., *Staph. aureus, Staph. epidermidis*	Thailand
Ika-Shiokara	Squid, salt	Fermented squid	*Micrococcus* sp., *Staphylococcus* sp., *Debaryomyces* sp.	Japan
Jaadi	Fish, salt	Salted fish		Sri Lanka
Karati, Bordia and Lashim	Fish (*Gudushia chapra, Pseudeutropius atherinoides,*	Side dish	*Lact. lactis, Leuc. mesenteroides, Lb. plantarum, B. subtilis, B. pumilus, Candida* sp.	India / Tamang et al., 2012

	Cirrhinus reba), salt			
Kung-chom	Shrimp, salt, sweetened rice	Fermented fish-rice	*Ped. cerevisiae*	Thailand
Kung chom	Shrimp(*Macrobrachum lanchesteri*), salt, garlic, rice	Fermented shrimp	*Ped. halophilus, Staphylococcus* sp., *Staph. aureus, Staph. epidermidis*	Thailand
Kusaya	Horse mackerel, salt	Fermented dried fish	*Corynebacterium kusaya, Spirillum* sp., *C. bifermentans, Penicillium* sp.	Japan
Myulchijeot	Small sardine, salt	Fermented small sardine	*Ped. cerevisiae, Staphylococcus* sp., *Bacillus* sp., *Micrococcus* sp.	Korea
Narezushi	Sea water fish, cooked millet, salt	Fermented fish-rice	*Leuc. mesenteroides, Lb. plantarum*	Japan
Nampla (Nampla-dee, Nampla-sod)	*Solephorus* sp. *Ristelliger* sp. *Cirrhinus* sp., Fresh water, brackish water, marine fish, salt	Fish sause	*Micrococcus* sp., *Pediococcus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Sarcina* sp., *Bacillus* sp., *Lactobacillus* sp., *Corynebacterium* sp., *Pseudomonas* sp., *Halococcus* sp., *Halobacterium* sp.	Thailand
Ngari	Fish (puntius sophore), salt	Fermented fish	*Lact. lactis, Lb. plantarum, Ent. faecium, Lb. fructosus, Lb. amylophilus, Lb. coryniformis, B. subtilis, B. pumilus, Micrococcus* sp., *Candida* sp., *Saccharomycopsis* sp.	India / Tamang et al., 2012
Patis	*Stolephorus* sp., *Clupea* sp., *Decapterus* sp., *Leionathus* sp., Fish, salt, food color-optional	Fish sauce	*Ped. halophilus, Micrococcus* sp., *Halobacterium* sp., *Halococcus* sp., *Bacillus* sp.	Philippines (Indonesia)
Pla-ra	Fresh water fish, salt, roasted rice	Fermented fish-rice	*Pediococcus* sp.	Thailand
Pla-chao (Pla-Khaomak)	Fresh water fish, salt, Khaomak	Thai sweetened fish	*Ped. cerevisiae, Staphyloccous* sp., *Bacillus* sp., *Micrococcus* sp.	Thailand
Pla-chom (Pla-khoa-kour)	Fresh water or marine anchovy, boiled rice, salt, garlic, roasted rice flour	Fermented fish, Thai anchovy	*Ped. cerevisiae, Lb. brevis, Bacillus* sp.	Thailand
Pla-paeng-daeng	Marine fish, red moulds rice (*Ang-kak*), salt	Red fermented fish	*Pediococcus* sp., *Ped. halophilus, Staph. aureus, Staph. epidermidis*	Thailand
Pla ra (Pla-dag, Pla-ha, Ra)	Fresh water fish, brackish water fish, marine fish	Fermented fish	*Ped. cerevisiae, Lb. brevis, Staphylococcus* sp., *Bacillus* sp.	Thailand
Pla-som (Pla-khao-sug)	Marine fish, salt, boiled rice, garlic	Fermented fish	*Ped. cerevisiae, Lb. brevis, Staphylococcus* sp., *Bacillus* sp.	Thailand
Saeoo Jeot (Jeotkal)	Shreimp(*Acetes chinensis*), salt	Fermented shrimp	*Halobacterium* sp., *Pediococcus* sp.	Korea

Som-fug (Som-dog, Pla-fu, Pla-muig, Fug-som)	Fresh fish, boiled rice, salt, garlic	Thai fermented fish	*Ped. cerevisiae, Lb. brevis, Staphylococcus* sp., *Bacillus* sp.	Thailand
Shottsuru	Anchovy, opossum shrimp, salt	Fish sauce	*Halobacterium* sp., *Aerococcus viridians (Ped. homari), halotolerant yeasts and halophilic yeasts*	Japan
Sidra	Fish (*Punitus sarana*)		*Lact. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. facalis, Ped. pentosaceus, W. confusus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp.	India / Tamang et al., 2012
Sikhae	Sea water fish, cooked millet, salt	Fermented fish-rice	*Leuc. mesenteroides, Lb. plantarum*	Korea
Suka ko maacha	River fish (*schizothorax richardsoni*), salt, tumeric powder	Eat as curry	*Lact. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp.	India / Tamang et al., 2012
Sukuti	Fish (*Harpodon nehereus*)	Pickle, soup and curry	*Lact. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp.	India / Tamang et al., 2012
Tai-pla	Fresh water fish, brackish water fish, marine fishes, salt	Fermented fish	*Pediococcus* sp., *Ped. halophilus, Staph. aureus, Staph. epidermidis*	Thailand

TABLE VIII

Representative traditional fermented fruit and root crop products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate	Sensory property and nature	Microorganisms	Country / references
Atchara	Green unripe papaya, onion, red pepper, garlic, ginger, salt	Unripe papaya pickle	*Leuc. mesenteroides, Lb. brevis, Lb. plantarum, Strep. faecalis, Ped. cerevisiae*	Philippines
Burong Mangga	Green unripe mango, salt	Pickled green mango		Philippines
Burong Prutas	Fruits, salt, sugar	Picked fruits	*Lb. brevis, Lb. plantarum, Leuc. mesenteroides*	Philippines
Ca Muoi	Round aubergine, galanga, salt	Fermented fruit	*Lactobacillus sp.*	Vietnam
Chikwangue	Cassava	Solid state	*Corynebacterium sp., Bacillus sp., Lactobacillus sp., Micrococcus sp., Pseudomonas sp., Acinetobacter sp. Moraxella sp.*	Central Africa; Zaire / Odunfa and Oyewole, 1997
Cingwada	Cassava	Solid state	*Corynebacterium sp., Bacillus sp., Lactobacillus sp., Micrococcus sp.*	East and Central Africa / Odunfa and Oyewole, 1997
Dage	Coconut press cake, Ragi		*Rhizopus sp.*	Indonesia
Fufu	Cassava	submerged	*Bacillus sp., Lb. plantarum, Leuc. mesenteroides, Lb. cellobiosus, Lb. brevis; Lb. coprophilus, Lact. lactis; Leuc. lactis, Lb. bulgaricus, Klebsiella sp., Leuconostoc sp., Corynebacterium sp., Candida sp.*	West Africa / Odunfa and Oyewole, 1997
Iape Ketela	Cassava, Ragi		*Rhizopus sp., Chlamydomucor sp., Candida sp., Saccharomyces sp., Endomycopsis sp.*	Indonesia
Gari	Cassava	Solid state	*Corynebacterium mannihot, Geotrichum sp., Lb. plantarium, Lb. buchnerri, Leuconsostoc sp., Streptococcus sp.*	West and Central Africa
Lafun / Konkonte	Cassava	Submerged	*Bacillus sp., Klebsiella sp., Candida sp., Aspergillus sp., Leuc. mesenteroides, Corynebacterium manihot, Lb. plantarum, Micrococcus luteus and Geotrichum candidum*	West Africa / Odunfa and Oyewole, 1997
Palm wine / Emu	Palm	Submerged	*Sacch. cerevisiae, Schizosaccharomyces pombe, Lb. plantarum, Leuc. mesenteroides*	West Africa / Odunfa and Oyewole, 1997
Tapai Ubi	Cassava, Ragi		*Chlamydomucor sp., Endomycopsis sp.,*	Malaysia

Tempoyak	Durian fruit	Fermented durian fruit	*Yeast, Bacillus sp., Acetobacter sp., Lactobacilus sp.*	Malaysian

TABLE IX

Representative traditional fermented milk products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate	Sensory property and nature	Microorganisms	Country / references
Arera	Cheese curd	Acid fermented butter milk	LAB	Ethiopia
Ayib	Goat milk		*Canida* sp., *Saccharomyces* sp., *Lactobacillus* sp., *Leuconostoc* sp.,	East and Central Africa / Odunfa and Oyewole, 1997
Buttermilk		Acid fermented butter milk	*Lb. bulgaricus*	Bulgaria
Chhu	Yak milk	Cheese like product	*Lb. farciminis, Lb. brevis, Lb. alimentarius, Lb. salivarius, Lact. lactis, Saccharomycopsis* sp., *Candida* sp.	India / Tamang *et al.*, 2012
Chhurpi (hard)	Yak milk	Chewable milk	*Lb. farciminis, Lb. casei, Lb. biofermentans, W. confusus*	India / Tamang *et al.*, 2012
Chhurpi (soft)	Yak milk	Cheese like product	*Lb. farciminis, Lb. paracasei, Lb. biofermentans, Lb.plantarum, Lb. curvatus, Lb. fermentum, Lb. alimentarius, Lb. kefir, Lb. hilgardii, W. confusus, Ent. faecium, Leuc. mesenteroides*	India / Tamang *et al.*, 2012
Curd	Milk	Yoghurt	*Lactobacillus* sp. *Sterptococcus* sp.	Sri Lanka
Dadhi	Cow milk, buffalo milk, sugar	Acid fermented butter milk	*Lactobacillus* sp. *Sterptococcus* sp.	Bangladesh
Dahi	Milk, starter culture	Yoghurt	*Lb. bifermentans, Lb. alimentarius, Lb. paracasei, Lact. lactis, Saccharomycopsis* sp., *Candida* sp.	Indonesia / Tamang *et al.*, 2012
Ergo	Milk	Acid fermented butter milk	LAB	Ethiopia
Jalebi	Wheat flour, dahi	Acid fermented milk	*Lb. fermentum, Lb. buchneri, Lact. lactis, Ent. faecalis, Sacch. cerevisiae*	India
Kefir	Goat, sheep, cow	Alcoholic fermented milk	*Tor. holmii, Tor. delbruechii, Lb. brevis, Lb. caucasicus,Strep. thermophilus, Lb. bulgaricus, Lb. plantarum, Lb. casei, Lb. brevis*	Russia / Rani and Soni, 2007; Bernardeau *et al.*, 2006
Kesong Puti, Keso, Kesiyo	Carabo's(buffalo) milk or cow carabao's milk, salt, Abomasal extracts coagulant, starter	White cheese, soft cheese	*Lb. helveticus, Lact. lactis, Lb. rhamnosus, Leuc. mesenteroides, Lb. acidophilus, Lb. plantarum, Lb. brevis, Lb. curvatus*	Philippines / Kisworo and Barraquio, 2003
Kishk	Milk, wheat	Fermented milk wheat mix	*Lb. plantarum, Lb. brevis, Lb. bulgaricus, Lb. casei, Strep.*	Egypt / Bernardeau *et al.*, 2006

			thermophilus	
Kushuk	Milk, wheat	Fermented milk wheat mix	*Lb. plantarum, Lb. brevis*	Iraq
Koumiss	Milk	Acid fermented milk	*Lb. bulgaricus, Torula sp., Lb. salivarius, Lb. buchneri, Lb. heveticus, Lb. plantarum, Lb. acidophilus*	Russia / Bernardeau *et al.*, 2006
Laban Rayeb	Milk	Acid fermented milk	*Lb. casei, Lb. plantarum, Lb. brevis, Lact. lactis, Sacch. kefir, Leuconostoc sp.*	Egypt / Bernardeau *et al.*, 2006
Laban zeer	Milk	Acid fermented milk	*Lb. casei, Lb. plantarum, Lb. brevis, Lact. lactis, Lact. lactis*	Egypt / Bernardeau *et al.*, 2006
Leben / Lben	Cow milk		*Candida* sp., *Saccharomyces* sp., *Lactobacillus* sp., *Leuconostoc* sp.,	North, East Central Africa / Odunfa and Oyewole, 1997
Liban-argeel	Sheep, goat, cow, buffalo milk	Acid fermented milk		Iraq
Philu	Cow or yak milk, bamboo vessels	Cream like product	*Lb. paracasei, Lb. bifermentans, Ent. faecium*	India / Tamang *et al.*, 2012
Rabdi	Maize flour, butter milk	Acid fermented milk	*Bacillus* sp., *Micrococcus* sp., *Ped. acdilactici, Pen. acidilactici*	India / Rani and soni, 2007; Blandino *et al.*, 2003
Somar	Yak or cow milk	Buttermilk	*Lb. paracasei, Lact. lactis*	India / Tamang *et al.*, 2012
Sour milk kerbah	Milk	Acid fermented milk	*Lact. lactis, Sacch. kefir, Lb. casei, Lb. brevis, Lb. plantarum*	Egypt
Sua chua	Dried skim milk, starter, sugar	Acid fermented milk	*Lb. bulgaricus, Strep. thermophilus*	Vietnam
Tahuri	Soybean milk	Soybean curd	LAB	Philippines
Trahanas	Milk, wheat	Acid fermented milk	*Strep. thermophilus, Lb. bulgaricus*	Greece

TABLE X

Representative traditional fermented meat products

(Tamang, 2010; Jung, 2012, amended and modified; other references are given in the table)

Product	Substrate	Sensory property and nature	Microorganisms	Country / references
Kargyong	Yak, beef, port, crushed garlic, ginger, salt	Sausage like meat product	*Lb. sakei, Lb. divergens, Lb. carnis, Lb. sanfrancensis, Lb. curvatus, Leuc. mesenteroides, Ent. faecium, B. subtilis, B. mycoides, B. thuringiensis, Staph. aureus, Micrococcus* sp., *Deb. hansenii, P. anomala*	India / Tamang *et al.*, 2012
Longanisa	Pork lean, pork backfat, ground pork, salt, sugar, soysauce, vinegar, anisado wine, potassium nitrate		LAB	Philippines
Nham (Musom)	Pork meat, pork skin, salt, rice, garlic	Fermented pork	*Pediococcus sp., Ped. cerevisiae, Lb. plantarum, Lb. brevis*	Thailand
Nem-chua	Pork, salt, cooked rice	Fermented sausage	*Pediococcus* sp., *Lactobacillus* sp.	Vietnam
Sai-krok-prieo	Pork, rice, garlic, salt	Fermented sausage	*Lb. plantarum, Lb. salivarius, Ped. pentosacuns*	Thailand
Satchu	Beef, yak, port, tumeric powder, edible oil, butter, salt	Ethnic dried meat	*Ped. pentosaceuous, Lb. casei, Lb. carnis, Ent. faecium, B. subtilis, B. mycoides, B. lentus, Staph. aureus, Micrococcus* sp., *Deb. hansenii, P. anomala*	India / Tamang *et al.*, 2012
Suka ko masu	Goat, buffalo meat, tumeric powder, mustard oil, salt	Dried or smoked meat	*Lb. carnis, Ent. faecium, Lb. plantarum, B. subtilis, B. mycoides, B. thuringiensis, Staph. aureus, Micrococcus* sp., *Debaromyces hansenii, P. burtonii*	India / Tamang *et al.*, 2012
Tapa	Lean beef, salt, sugar, potassium nitrate	Semi-fermented thinly sliced beef		Philippines
Tocino	Pork, salt, sugar, potassium nitrate	Fermented cured pork	*Ped.* cerevisiae, Lb. brevis, *Leuc.* mesenteroides	Philippines

ANNEX 2

CASE STUDY
Asia: Himalayan traditional fermented foods

Thoughts and visions for the Himalayan region have been communicated by Prof. Jyoti Prakash Tamang [Department of Microbiology, Sikkim University (National University), 6th Mile, Tadong 737102, Gangtok, Sikkim, India], which are herewith gratefully acknowledged.

Possible prospects and lines of approach towards development and commercialisation can be outlined on the basis of the following important parameters:

- **Microbial genetic resources**

There is an increased awareness of microbial diversity and the importance of microbes as genetic resources for diverse functional applications in food production, medicine, agriculture and environment management. As ecosystems, food bioresources constitute a basic part of the human environment where, by traditional abilities and skills, mankind selected edible and culturally acceptable foods from available natural resources. Ethnic fermented foods and beverages are an important source of microbial diversity in the Himalayas. Most of the ethnic fermented foods and beverages collected from different places in the Himalayas have been extensively studied (see Tables II, IV and IV in particular), and the functional microorganisms associated with ethnic foods were isolated, characterized and identified (Batra and Millner, 1976; Chettri and Tamang, 2008; Dewan and Tamang, 2006, 2007; Jeyaram *et al*., 2008 a, b; 2011; Karki *et al*., 1983; Nikkuni *et al*., 1996; Oki *et al*., 2011; Rai *et al*., 2010; Sarkar *et al*., 1994; 2002; Shrestha *et al*., 2002; Tamang, 1999, 2003; Tamang *et al*., 2000, 2002, 2005, 2007, 2008, 2010; Tamang and Nikkuni, 1996; Tamang and Thapa 2006; Tamang and Sarkar 1993, 1995, 1996; Tamang and Tamang, 2007, 2009, 2010; Thapa *et al*., 2004, 2006, 2007; Thapa and Tamang, 2004, 2006; Tsuyoshi *et al*., 2005; Yonzan and Tamang, 2010).

Identification of isolated strains served to reflect the species diversity of microbial communities associated with traditional fermented foods of the Himalayas and beverages, as listed below:

- **LAB**: *Enterococcus faecium, Ent. faecalis, Ent. durans, Ent. durans, Ent. hirae; Lb. alimentarius, Lb. amylophilus, Lb. bifermentans, Lb. brevis, Lb. buchneri, Lb. bulgaricus, Lb. carnis, Lb. coryniformis* subsp. *torquens, Lb. curvatus, Lb. delbrueckii, Lb. divergens, Lb. farciminis, Lb. fructosus, Lb. hilgardii, Lb. kefir, , Lb. plantarum, Lb. paracasei* subsp. *pseudoplantarum, Lb. paracasei* subsp. *paracasei, Lb. salivarius, Lb. sakei, Lb. sanfransiscensis; Lact. lactis, Lact. lactis* subsp. *lactis, Lact. lactis* subsp. *cremoris Lact. plantarum; Leuc. fallax, Leuc. lactis, Leuc. mesenteroides, Leuc. citreum; Ped. pentosaceus, Ped. acidilactici; Tetr. halophilus; W. confusa, W. cibaria.*
- **Bacilli**: *B. cereus, B. circulans, B. coagulans, B. laterosporus, B. lentus, B. licheniformis, B. mycoides, B. pumilus, B. sphaericus, B. subtilis, B. thuringiensis.*
- **Other bacteria**: *Alkaligenes* spp., *Micrococcus* spp., *Staph. aureus, Staph. sciuri.*
- **Yeasts**: *Cand. bombicola, Cand. castellii, Cand. chiropterorum, Cand. glabrata, Cand. famata, Cand. parapsilosis, Cand. montana, Cand. tropicalis, Cand. versatilis*; *Deb. hansenii, Deb. polymorphus, Deb. pseudopolymorphus; Ge. candidum; Pi. anomala, Pi. burtonii, Pi. fabianii, Pi. guilliermondi; Sacch. cerevisiae, Sacch. bayanus, Sacch. kluyveri*; *Sm. capsularis, Sm. crataegensis, Sm. fibuligera; Zygosaccharomyces rouxii.*
- **Moulds**: *Asp. oryzae*; *Mu. circinelloides, Mu. hiemalis; Rhiz. chinensis, Rhiz. oryzae, Rhiz. stolonifer* var. *lyococcus.*

Some of these strains possess protective and functional properties, rendering them potential candidates for use as starter culture(s) for controlled and optimized production of traditional fermented foods with improved quality. Information on the characteristics of the microorganisms isolated from the ethnic fermented foods significantly enriches the database of microbial diversity from food ecosystems in the Himalayas. Besides being proposed for starter cultures, some strains can be exploited for production of enzymes, bioactive compounds and other industrial uses. It would be worthwhile to pursue the establishment of a gene bank in the Himalayas for preservation and maintenance of these strains, and for the purpose of further investigations.

- **Interpretation of "Ethno-Microbiology" in the Himalayas**

Ethnic fermented foods and beverages have importance in the context of the Himalayan food eco-systems, and in terms of culture, tradition, cost-effective production and nutrition. Due to cultural adaptation for consumption, and the preserved nature of the products, the majority of the Himalayan fermented foods can be considered essential for food and nutritional security of the region (Tamang *et al*., 2010). The Himalayan people contribute their traditional knowledge of the production and management of available food bioresources, which, in turn, supplements the food ecosystem and enhances the regional economy. Based on this model, some of the traditional food commodities can be commercialized in the Himalayan regions.

- The native skills of alcohol production by 'starter culture' technique and using traditional distillation apparatus is well recognized. The consortium of microorganisms (starter culture) is preserved in a rice or wheat base, as a source of starch, together with the use of glucose-rich wild herbs to supplement the carbon sources for growing microorganisms (Tamang, 2010).
- Unlike mixed culture starters of the other Asian countries, *marcha* is usually prepared by wrapping kneaded dough cakes in fern fronds with the fertile side touching them. This may be due to abundance of ferns locally called 'pire uneu' {*Glaphylopteriolopsis erubescens* (Wall ex Hook.) Ching} in the Himalayas. Probably, germination of spores in sori helps to maintain the warmth of the fermenting mass in cold climates. Preparation of *marcha* is similar to those of other starter cultures of Asia. *Marcha* makers believe that addition of wild herbs give more sweetness to the product. Addition of chillies and ginger during *marcha* preparation is to prevent growth of undesirable microorganisms that may inhibit the growth of native functional microorganisms in *marcha*. Studies of Soedarsono (1972) in *ragi*, an Indonesian rice-based starter culture, reveal that certain spices inhibit many undesirable microorganisms at the time of fermentation. Hesseltine (1983) speculated that the spices, which are known to be inhibitory to many bacteria and moulds, are the agents that select the right population of microorganisms for fermentation. *Marcha* making technology reflects the traditional method of sub-culturing desirable inocula from previous batch to new culture using rice as base substrates. This technique preserves the microbial diversity essential for beverages production. *Marcha* retains its potency *in situ* for over a year or more.
- Diversity of fermented foods is related to diversity of ethnicity with unparallel food culture of each community. Microbial diversity ranges from filamentous moulds to enzyme-producing to alcohol-producing yeasts, and Gram-positive and few Gram-negative bacteria with several functional properties. There is a relationship between the human life and microorganisms. Ethnic fermented foods and beverages have biological functions enhancing several health-promoting benefits to the consumers due to functional microorganisms associated with them. It has been noticed that many minor but culturally important ethnic foods are not seen in local markets, and majority of the young generations do not know ethnic foods, their culinary practices and processing method. Native microorganisms with vast biological importance and potential genetic resources which are

associated with ethnic fermented foods are forced to disappear. Survey on consumption and production of ethnic fermented food and beverage across the world and calculation of *per capita* consumption is an urgent need to be addressed by the food policy makers. Introduction of ethnic fermented foods in the syllabus at the master level in Microbiology, Food Sciences of all Universities may be initiated. Sikkim University has already incorporated the separate paper on ethnic fermented foods and beverages of the world in Microbiology at graduate level.

- **Restoration of "ethno-microbiology" practices in the Himalayas by women**

Some villages in the Himalayas, in the Darjeeling hills, Sikkim and East Nepal, involve traditional microbiological systems for *marcha*-making, and can be considered as centres of microbial and genetic resources. These villages during a survey (Tamang, 2010) have been identified as the possible microbial resources necessary for ethnic alcohol production. The Himalayan women have been sub-culturing and maintaining a consortium of functional microorganisms for alcohol production in the form of dry ball-flatted cake like starter called *marcha* (see also 3.4) or *phab* for more than 1500 years (Tamang, 2010). Some of the important *marcha*-making villages in the Eastern Himalayas are: Nor Busty (Darjeeling: dominant *marcha*-makers women - Rai, Limboo); Kashyong (Kalimpong: Limboo, Rai); Mangzing (Kalimpong: Limboo, Rai, Lepcha); Jhosing (North Sikkim: Limboo); Tibuk (North Sikkim: Limboo); Chhejo (West Sikkim: Limboo); Lingchom (West Sikkim: Limboo); Salghari (South Sikkim: Limboo); Barnyak (South Sikkim: Limboo, Rai); Aho (East Sikkim: Limboo); Kopchey (East Sikkim: Limboo); Change (Taplejung-Nepal: Limboo); Ahale (Bhojpur-Nepal); Bokhim (Bhojpur-Nepal: Gurung); Rajarani (Dhankuta-Nepal: Limboo); Nundhaki (Sankhuwasabha-Nepal: Limboo); Terhathum (Terhathum-Nepal: Limboo). It has been recommended (Tamang, 2010) that these villages should be preserved (culturally protected) and the rural women processors involved be trained in basic microbiological and hygiene principles as a basis for understanding the principles and role of sub-culturing and preserving for ensuring the characteristic fermentation.

- **Traditional technology for making *Kinema***

There is a good market for *kinema*, a fermented soybean food in India, Nepal and Bhutan, similar to other *Bacillus*-fermented soybean foods of Asia (see Table II on legume fermentations), and it provides a reliable source of income to many rural women. Still, *kinema* processing has not been included yet in the loan-scheme of public sector banks or financial institutions, neither incorporated in the rural development programme and cottage-level industry scheme of any local government in the Eastern Himalayas. Ready-to-use pulverised starter developed for *kinema* production has been developed by Tamang (1999) and can be introduced to *kinema* makers, after adoption to local conditions, as a cost-effective source of income generation.

- **Medicinal Aspects**

The food habits of the Himalayas usually do not require additional medicines or supplementary drugs. Most of the ethnic foods, both fermented and non-fermented, have therapeutic values and eaten for prevention of illness. Traditionally the Himalayan ethnic people do not have habits of taking drugs and medicines in forms of tablets, tonics, etc. (Tamang, 2010). This may be due to therapeutic values of their ethnic fermented foods such as antioxidant, antimicrobial, probiotics, low-cholesterol, essential amino acids, bio-nutrients, and some important bio-active or health-benefits compounds (Tamang, 2007). It is commonly believed that their foods have medicinal properties. *Somar* is used to cure stomach aches, and to control frequent diarrhoea by the Sherpa. *Gundruk* and *sinki* are used during indigestion and commonly eaten as appetizer. The Meitei believes that *soibum* has wound healing and anti-tumour properties for animals, particularly cows. *Kinema* is highly nutritive and has several health-promoting benefits (Tamang *et al.*, 2009a; Tamang, 2010; Omizu *et al.*, 2011). *Jaanr* and other ethnic fermented beverages are high calorie foods, and also rich in minerals and vitamins, mostly given

to post-natal women. *Bhaati jaanr* and *poko* promote good health, nourish the body giving good vigour and stamina. It is also suggested that ethnic Himalayan milk products have protective and probiotic properties which stimulate the immune system, cure stomach-related diseases (Dewan and Tamang, 2006, 2007; Tamang *et al*., 2000, 2009a,b). *Raksi* is a stimulating alcoholic drink which has both social and medicinal importance in the food culture of the Himalayan people. Though clinical study of the Himalayan fermented foods have yet to be conducted, people have customary belief in therapeutic values of some of their foods, which have been in use both as foods and as therapy for centuries. Such ethnic foods, if studied properly, may be presented even in global markets.

- **Commercialisation through Ethnic Food Tourism**

Occasional visits by tourists to new places and countries are becoming popular all over the world. Tourist destinations in a few hill resorts in the Himalayas are increasing year by year. The concept of 'ethnic food tourism' may have relevance in the present days due to the growth of the tourist industry in the Himalayas. Interaction with the people of other regions/countries, exploring their traditional values and culture, will serve to link the enjoyment of dining to the local culture. Ethnic values of food harness the cultural history of a community, their valuable indigenous knowledge of food production, vast nutritious qualities, functional microorganisms, regional economy, and the enjoyment of dining at local cuisines (Tamang, 2010).

The major components of food tourism are the tourism food product, consumer behaviour, and ethnic food production sites relevant for local market potential, and with traditional food as an attraction in destination marketing (Hall *et al*., 2003). France attracts the highest annual number of tourists worldwide, reaching 79.5 million (UNWTO 2012). A major reason could be the high quality food and wine, served in inexpensive traditional restaurants that offer the local agricultural produce to millions (Kimura, 2000). The main cuisine of France is cheese and wine, both fermented products. There are more than 5000 restaurants in Paris serving the traditional dishes along with French wine. In France, tourists can visit the vineyards, and watch how wines are manufactured, also with the opportunity to sample a number of different kinds of wines, involving the vineyard and a trip that revolves around wine tourism. The Himalayan ethnic foods can be diversified into more presentable forms to attract the tourists to taste the aroma, flavour and texture of the unique recipes. Promotion of food tourism will stimulate the opening of more ethnic food restaurants, food huts and stalls, which would focus on traditional utensils, kitchen wares, traditional culinary and customs and advocate the importance of ethnic values of the fermented foods. Tourists may be encouraged to visit villages where they can enjoy and learn the traditional processing of the Himalayan foods like in a vineyard in France, *tempeh* production in Indonesia, *shoyu* and *sake* manufacturing in Japan, etc. Possible sites for visiting the traditional food processing in the Himalayas are: *kinema* production at Aho village in Sikkim, *kargyong* and hard-variety *chhurpi* making in North Sikkim, pit fermentation of *sinki* production at Kalimpong; *marcha*-making in Therathum village in Nepal; *chyang* production in Ladakh, *ngari* production in Manipur, *mohi* production in Nepal, etc. Village tourism has been increasing in the Himalayas. Serving the standardised ethnic Himalayan foods and drinks to tourists and travellers in village resorts will not only boost the regional economy but also enhance an increase in local agricultural and livestock production due to constant demand, at the benefit of marginal farmers and local sellers. Unlike in urban tourism, tourists in villages shall get the chance to learn more about the culinary skills of the local people and promote the Himalayan foods outside the regions.

ANNEX 3

Examples of genetically engineered microorganisms and their applications in food processes

BOX 3 ***Saccharomyces cerevisiae***

As a model for understanding eukaryotic organisms and a cell factory in classical and modern biotechnological processes, the yeast *Saccharomyces cerevisiae* is of great importance. Its broad use in industry is closely related to its role as a major platform for metabolic engineering, which aims to enhance yeast biotechnology. This underpins baking, brewing and wine making, as well as the microbial production of a growing number of other interesting compounds with various applications. For example, numerous specialised *Sach. cerevisiae* wine strains were obtained in recent years, possessing a wide range of optimized or novel oenological properties, capable of satisfying the demanding nature of modern winemaking practice. The unlocking of transcriptome, proteome and metabolome complexities will contribute decisively to the knowledge about the genetic make-up of commercial yeast strains and will influence wine strain improvement via genetic engineering (Bisson *et al.*, 2007). Former metabolic engineering approaches were primarily rational, i.e. based on available knowledge about the metabolic pathways and enzymes involved. A more recent alternative is inverse metabolic engineering, for which the phenotype is the starting point. The goal is to exploit natural variability and analyse the molecular basis of various manifestations of a trait, thereby elucidating novel targets for strain improvement (Nevoigt, 2008; Schuller and Casal, 2005).

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