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Full-length Review

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FULL-LENGTH REVIEW – Food Microbiology

# The forgotten role of food cultures

François Bourdichon[1,2,*,†], Emmanuelle Arias[3], Andrzej Babuchowski[4], Anne Bückle[5], Fabio Dal Bello[6], Aurélie Dubois[7], Alessandra Fontana[2], Duresa Fritz[8], Rober Kemperman[9], Svend Laulund[10], Olivia McAuliffe[11], Marta Hanna Miks[12,13], Photis Papademas[14], Vania Patrone[2], Dinesh K. Sharma[15], Edward Sliwinski[16], Catherine Stanton[11], Ueli Von Ah[3], Su Yao[17] and Lorenzo Morelli[2]

[1]Food Safety, Microbiology, Hygiene, 16 Rue Gaston de Caillavet, 75015 Paris, France, [2]Facoltà di Scienze agrarie, alimentarie ambientali, Università Cattolica del Sacro Cuore, Via Emilia Parmense, Piacenza-Cremona, Italy, [3]AGROSCOPE, Food Microbial Systems, Schwarzenburgstrasse 161, CH-3003 Bern, Switzerland, [4]Dairy Industry Innovation Institute, Kormoranow St., Mragowo 11–700, Poland, [5]Milchprüfring Baden-Württemberg e.V., Marie-Curie-Straße 19, 73230 Kirchheim, u.T., Germany, [6]Sacco S.r.l., Via Alessandro Manzoni, Cadorago (Co), Italy, [7]International Dairy Federationiry Federation, 70 Boulevard Auguste Reyers, 1030 Brussels, Belgium, [8]International Flavors and Fragrances, 20 rue Brunel, Paris 75017, France, [9]Lesaffre International, 152 rue du Docteur Yersin, 59120 Loos, France, [10]Chr. Hansen A/S, Agern Allé 24, 2970 Hoersholm, Denmark, [11]Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland, [12]Glycom A/S, Kogle Allé 4, 2970 Hørsholm, Denmark, [13]Faculty of Food Science, Food Biochemistry, University of Warmia and Mazury in Olsztyn, Plac Cieszynski 1, 10–726 Olsztyn, Poland, [14]Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Archiepiskopou Kyprianou, PO BOX 50329, Limassol, Cyprus, [15]National Dairy Development Board, Anand 388001, India, [16]The European Federation of Food Science & Technology, Nieuwe Kanaal 9a, 6709 PA, Wageningen, The Netherlands and [17]China National Research Institute of Food & Fermentation Industries, China Center of Industrial Culture Collection, Building 6, No.24, Jiuxianqiaozhong Road, Chaoyang District, Beijing 100015, PR China

[*]**Corresponding author:** Food Safety, Microbiology, Hygiene, 16 Rue Gaston de Caillavet, 75015 Paris, France. Tel: +33 6 24 75 21 49; E-mail: francois.bourdichon@gmail.com

**One sentence summary:** Perspective on the potential of food cultures for the biopreservation of food products and applications to limit food waste.

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[†]François Bourdichon, http://orcid.org/0000-0003-0373-0407

## ABSTRACT

Fermentation is one of if not the oldest food processing technique, yet it is still an emerging field when it comes to its numerous mechanisms of action and potential applications. The effect of microbial activity on the taste, bioavailability and

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preservation of the nutrients and the different food matrices has been deciphered by the insights of molecular microbiology. Among those roles of fermentation in the food chain, biopreservation remains the one most debated. Presumably because it has been underestimated for quite a while, and only considered – based on a food safety and technological approach – from the toxicological and chemical perspective. Biopreservation is not considered as a traditional use, where it has been by design – but forgotten – as the initial goal of fermentation. The 'modern' use of biopreservation is also slightly different from the traditional use, due mainly to changes in cooling of food and other ways of preservation, Extending shelf life is considered to be one of the properties of food additives, classifying – from our perspective – biopreservation wrongly and forgetting the role of fermentation and food cultures. The present review will summarize the current approaches of fermentation as a way to preserve and protect the food, considering the different way in which food cultures and this application could help tackle food waste as an additional control measure to ensure the safety of the food.

**Keywords:** food cultures; fermentation; biopreservation; food safety; regulation; mechanism of action

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## INTRODUCTION

Food preservation has been a key concern since the earliest days of humanity. Among the numerous empirical processes that have been developed and passed down, fermentation is one of the oldest preservation techniques and still widely used in various food matrices. Fermentation produces beneficial effects in foods that undergo chemical changes caused by microorganisms such as bacteria or yeasts (Caplice and Fitzgerald 1999).

Fermentation plays different roles in food processing. Major roles considered are as follows:

- Preservation of food through formation of inhibitory metabolites such as organic acid (lactic acid, acetic acid, formic acid, andpropionic acid), ethanol, bacteriocins and so on, often in combination with decrease of water activity (by drying or use of salt; Ross, Morgan and Hill 2002; Gaggia *et al*. 2011.
- Improving food safety through inhibition of pathogens (Adams and Mitchell 2002, Adams and Nicolaides 2008) or removal of toxic compounds (Hammes and Tichaczek 1994.
- Improving the nutritional value (Poutanen, Flander and Katina 2009, van Boekel *et al*. 2010.
- Organoleptic quality of the food (Marilley and Casey 2004; Smit, Smit and Engels 2005; Lacroix *et al*. 2010; Sicard and Legras 2011).

Biopreservation is a natural way to protect against spoilage and harmful contamination in food. This helps keeping food products fresh and safe throughout shelf life, opening for the possibility of reducing food waste. The food industry is presently looking for means of producing safe food products with an extended shelf life thus reducing food waste and meeting the consumer demands for natural, low salt, low sugar foods and for reduced use of chemical preservatives. Fermented food products have a longer shelf life and are less prone to spoilage than fresh food products of the same matrix (e.g. cheese compared to milk). There have been advances in the understanding of food microbiology, and the ability to screen for food cultures with better ability to stabilize food provides food cultures with bioprotective effect (Bech Hansen 2002). The advances of science in this field are eventually restricted by regulations in place, as this protective role of food cultures is mistakenly assimilated to the category of food additives and is not considered as a traditional use of food cultures in the food chain (Laulund *et al*. 2017). The aim of the present review is to highlight how the use of food cultures is already protecting the fermented food products, with enhanced shelf life and reduced spoilage.

The microbial stability and safety as well as the sensory and nutritive quality of foods are achieved by applying a combination of several different preservative factors called hurdles. The most important hurdles for keeping food fresh and safe are temperature (high or low), water activity ($a_w$), acidity (pH), redox potential, preservatives (food additives), competitive microorganisms (bacteria, moulds and yeasts) and their metabolites. The competitive microorganisms with enhanced protective effect can be indigenous or be added as specifically selected food cultures.

As proposed by the International Dairy Federation on its factsheet on the topic (Available at: https://fil-idf.org/publications/free-of-charge/idf-factsheet-007–2019-bioprotection/) Biopreservation refers to enhanced food safety and extended shelf life of foods by indigenous and/or intentionally added microbiota, inhibiting growth of pathogenic and spoilage organisms due to microbiological competition and production of antimicrobial metabolites. Among the different species of food cultures, lactic acid bacteria have a major potential for use as biopreservation supported by their long history of safe use, proven antimicrobial properties, their capacity to naturally dominate the microflora and occupy the ecological niche during storage.

Food cultures for fermentation do all have biopreservation effects due to metabolically activities. While the traditional use of cultures in fermented foods refers to their positive action on product properties (texture, aroma, digestibility, …), more specific focus is now on food cultures with appropriate biopreservative properties for a given application, inhibition of the spoilage microflora and improvement of food safety.

Biopreservation enhances the effectiveness of a food management system, but is never an alternative to good cleaning practices, hygienic design of the production and cold chain conservation (Motarjemi and Nout 1996). Food cultures with biopreservative effect create an extra protective hurdle against specific pathogens and/or spoilage microorganisms in the product during processing and/or after the product has left the manufacturing facility, e.g. during transport, storage, retail display and even after opening of the packaging by the consumer (Baka et al 2014).

The selection and application of protective food cultures must comply with the same safety criteria as for all food cultures used in the food industry. Food cultures are chosen for their ability to control and reduce foodborne pathogens and spoilage microorganisms by exploiting microbial competition and dominance phenomena. Isolation, selection, detailed characterization and validation of cultures are a way of taking advantage of the natural way in which microorganisms compete with each other in a complex environment in order to ensure that the added biopreservative food culture has the specific characteristics under specific condition(s). This allows more control over the process than with spontaneous fermentation. A spontaneous fermentation is actually also what takes place in fresh and ready to eat (RTE) foods resulting in spoilage New analytical tools make it feasible to identify and characterize microorganisms

present in a given environment. These enable the selection of the best candidates from a very high number of food cultures to protect against pathogenic and spoilage microorganisms. Individual food culture strains within the same species have different fermentation properties that can create variations in taste, aromas and texture/viscosity that may or may not be wanted. A careful selection of individual strains can be based on well-known and commonly used species, but have to be tested individually regarding properties contributing to fit the purpose of certain biopreservative effects and possible side effects (Bourdichon *et al.* 2012).

## GENETIC BASIS AND PRODUCTION OF SPECIFIC METABOLITES AND/OR ENZYMES

Microbes have the ability to produce an extraordinary array of metabolic by-products exhibiting a bactericidal or bacteriostatic activity such as organic acids, enzymes, bacteriocins and many other secondary metabolites. Biopreservation involves applying food-grade microorganisms to extend the shelf life of foods, and preventing the development of undesirable microorganisms (Elsser-Gravesen and Elsser-Gravesen 2014).

## GENERAL AND LOW MOLECULAR WEIGHT METABOLITES

The end products of fermentation may include organic acids (lactate, acetate, succinate and formate), gases ($CO_2$, $H_2$ and $SO_2$), hydrogen peroxide and other metabolites (ethanol, diacetyl, aldehydes, ketones, fatty acids and so on). The proportions of these end products vary between and within species. Many of these metabolites display antagonistic effects against other microbes through various modes of action (Ben Said *et al.* 2019). Examples include $CO_2$ which has long been documented as inhibitory to certain pathogenic species. Heterofermentative lactic acid bacteria (LAB) species produce $CO_2$ from formate, creating an anaerobic environment in the food which is hostile to aerobic species. Hydrogen peroxide ($H_2O_2$)-related inhibition occurs through oxidative damage of proteins and, at times, an increased membrane permeability take place in the target organisms. Another example is diacetyl (2,3-butanedione), produced by certain LAB species. While diacetyl can be employed as a biopreservative, its contribution to the flavor and aroma of a product means that it has limited preservative uses in foods where diacetyl is not wanted ad a major flavor component. (Drosinos *et al.* 2005).

## ANTIFUNGAL COMPOUNDS

Antifungal compounds are key to avoiding spoilage with yeasts and/or moulds, and a recent review describes the role and use of LAB as bioprotective food cultures against fungal spoilage in foods (Siedler, Balti and Neves 2019). Some compounds previously discussed, e.g. certain organic acids such as propionic acid, and reuterin can be used to inhibit fungi as well as bacteria (Corsetti *et al.* 1998, Cleusix *et al.* 2007). Phenolic acids produced by *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) are involved in the antifungal activity or in the reduction of aflatoxin production in e. g. maize (Nazareth *et al.* 2020). In other cases, antifungal compounds produced by LAB are peptides by chemical nature, such as those against *Aspergillus flavus* on maize (Muhialdin *et al.* 2020). Phenyllactic acid is another compound that can be used to inhibit both bacteria and fungi. It is present in different forms such as 3-phenyllactic acid and 4-phenyllactic acid. Both are produced by LAB (Ström *et al.* 2002, Mu *et al.* 2010), whereas 3-phenyllactic acid can also be produced by *G. candidum* (Dieuleveux, Lemarinier and Guéguen 1998). In the latter case, the inhibition can also be towards *Fusarium* spp. and reducing the toxin concentration (Kawtharani *et al.* 2020). Since phenyllactic acid is produced by many LAB, it is often also naturally present in fermented foods and can be used in a variety of food products.

## KILLER YEASTS

The multitude of fermented bacterial substances and mechanisms to control growth of other microorganisms, are similar known from yeasts fermentation. Killer yeasts produce and excrete extracellular proteins which are lethal to sensitive strains of other yeasts or other microorganisms (Starmer *et al.* 1987). These killer toxins (mycocins) were first detected in *Saccharomyces cerevisiae* and later in strains of other species such as *Debaromyces* spp., *Kluyveromyces* spp., *Pichia* spp., *Metschnikowia* spp. as well (reviewed in Mannazzu *et al.* 2019). Killer yeasts are applied as biocontrol agents especially in winemaking to control indigenous yeasts which produce offflavors (reviewed in Morata *et al.* 2020). Moreover, they have been used as bioprotectors in vegetables, olives, beer, sake, dry-cured ham, sausages, yogurt and cheese (reviewed in Salas *et al.* 2017, Medina-Córdova *et al.* 2018, Guimaraes *et al.* 2018, Mannazzu *et al.* 2019).

## BACTERIOCINS

Bacteriocins are ribosomally synthesized peptides or proteins that possess antimicrobial activity towards closely related bacterial species, whereas the producing bacterium is immune to the specific bacteriocin itself (De Vuyst and Leroy 2007).

As examples, nisin has been chosen among lantibiotics (Class I bacteriocins), and lactococcin G and pediocin A among nonlantibiotics (Class II bacteriocins).

### Nisin

Different strains of *Lactococcus lactis* subsp. *lactis* are known as bacteriocin producers. One of the most intensively studied and used is the lantibiotic nisin. Nisin is an effective agent against several undesirable Gram-positive bacteria in cheese and various other foods. It was also the first antimicrobial peptide approved by the FDA to be utilized as a food additive preservative (De Vuyst and Vandamme ). The list of FDA approved food additives and GRAS (generally recognized as safe) notices with intended antimicrobial effect is provided in Table 1 (As available on December 2020–Available at: https://www.fda.gov/food/gras-notice-inventory/recently-published-gras-notices-and-fda-letters).

Nisin is a ribosomally synthesized lantibiotic and many variants have been discovered, naturally produced by several strains of *L. lactis* (i.e. Nisin A, Z, Q, F) and *S. uberis* (i.e. Nisin U, U2; Gross and Morell 1971; de Vos *et al.* 1993; Zendo *et al.* 2003; Wirawan *et al.* 2006; de Kwaadsteniet, Ten Doeschate and Dicks 2008). The most common variants are nisins A and Z, which differ in one amino acid residue. The biosynthesis and regulatory machineries of lantibiotics are encoded by genes organized in operons. Single genes can vary their location within the operon, but invariably they are clustered on the genome. For bacteriocin synthesis up to three functions are required: production, immunity and, optionally, quorum sensing. In the case of

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**Table 1.** List of FDA approved food additives and GRAS (generally recognized as safe) notices with intended antimicrobial effect.

| Food additives | | |
|---|---|---|
| | **Intended use** | **Food matrix** |
| Acetic acid | Buffer and neutralizing agent | Cheese |
| Hydrogen peroxide | Used in combination with acetic acid to form peroxyacetic acid | Wash water for fruits and vegetable that are not raw agricultural commodities (59 ppm) |
| Lactic acid | Buffer and neutralizing agent | Bakery products, cheese, frozen desserts, fruit butters, jellies and preserves |
| Propionic acid | Preservative | Swiss and Gruyere Cheese |
| Natamycin (pimaricin) | Antimycotic | Cheese (< 20 mg/kg finished product) |
| Nisin preparation | Antimicrobial | Cheese (< 250 ppm) |
| **GRAS-Microorganisms** | | |
| *Lactobacillus curvatus* DSM 18775 | Antimicrobial (*Listeria monocytogenes*) | Ready-to-eat cooked meat, poultry products |
| *Lb. acidophilus, Lb. lactis* and *Pediococcus acidilactici* | Antimicrobial | Meat, poultry |
| *Carnobacterium maltaromaticum* CB1 (viable and heat-treated) | Antimicrobial (*Listeria monocytogenes*) | Various foods |
| *Carnobacterium maltaromaticum* CB1 | Antimicrobial (*Listeria monocytogenes*) | Ready-to-eat meat products |
| **GRAS-Bacteriocins** | | |
| Bacteriocin preparations specific to Salmonella | Antimicrobial (Salmonella) | Red meats, poultry and egg products (max. application rate 3 mg/kg or L) |
| Colicin preparations | Antimicrobial | Meat (application rate of 1–10 mg/kg) |
| Natamycin | Antimycotic (yeasts and molds) | Yogurt (levels < 5 ppm finished product) |
| Nisin | Antimicrobial | Casings of frankfurters; cooked meat; poultry products |

nisin, these functions are scattered across the nisin biosynthesis gene cluster nisABTCIPRKFEG which consists of 11 genes divided into four operons (Lubelski *et al.* 2008). Bacteriocin production is encoded by nisABTC and nisP, nisA encoding the bacteriocin precursor, nisT the transporter exporting the unmodified precursor, nisBC encoding post-translational modification functions and nisP encoding the leader peptidase (Kuipers *et al.* 1993). Immunity is encoded by nisI and nisEFG, a dedicated immunity protein and an ABC-transporter, respectively (McAuliffe, Ross and Hill 2001). Lastly, the two-component signal transduction system responding to the mature nisin consisting of a response regulator and histidine kinase is encoded by nisRK, respectively (Ge *et al.* 2017). The self-protection mechanism of the food culture producers can involve more than one system. Comparative genomic analyses by Wels and collaborators (Wels *et al.* 2019) on publicly available genome sequences of *L. lactis* subsp. *lactis* and subsp. *cremoris* revealed a complete nisin biosynthesis cassette nisABTCIPRKFEG (Kuipers *et al.* 1993) on the chromosome of the subspecies *lactis* strains CV56 and IO-1. This gene cluster is flanked by transposase fragments. A complete nisin gene cluster was also found in the subspecies *lactis* strains KF134, KF146, KF196, KF282, K231, KF24, K337, KF67, KF7, Li-1 and LMG8526, at the same chromosomal insert position as in strain CV56. It was highlighted that all the strains of plant or vegetable origin can produce nisin Z. On the contrary, the subspecies *cremoris* strain V4 and the subspecies *lactis* strains LMG14418, LMG9446 and KF147 present an incomplete chromosomal gene cluster and cannot produce nisin, but they have maintained some immunity genes (i.e. nisFEG and/or nisI) (Wels *et al.* 2019). *Lactococcus lactis* ssp. *cremoris* FG2 and N41, from dairy starter and soil/grass, respectively, present a partial nis gene cluster encoding only nisP, nisI and a truncated nisC. This organization suggests a localization typical of plasmid (Tarazanova *et al.* 2016). The original nisin gene cluster is chromosome based. The detection of a plasmid based nisin variant produced by *Streptococcus capitis* (O'Sullivan *et al.* 2020) and the detection of nisin variants in multiple species suggest that nisin like gene clusters can also be horizontally transferred on mobile elements.

*Lactococcus lactis* cheese starter cultures may either produce and/or tolerate the antimicrobial bacteriocin nisin. It is therefore relevant upon the choice of food cultures to assess their natural resistance or sensitivity to potentially produced bacteriocins (Van Gijtenbeek *et al.* 2021).

## Pediocin PA-1

Pediocin, produced by *Pediococcus acidilactici*, belongs to the class II bacteriocins (nonlantibiotics), a large and diverse group of antimicrobial compounds that includes small heat-stable, cationic and hydrophobic/amphiphilic peptides. They are mainly active against other LAB, and they damage target cells by pore formation or by interfering with the integrity of the membrane (Nes *et al.* 1996). Though most have a limited activity spectrum, some, including pediocin PA-1, also inhibit more distantly related bacteria. The key interest in pediocin PA-1 in relation to biopreservation it its functionality in inhibition of the pathogen *Listeria monocytogenes*. Where the nisin biosynthetic gene cluster is an example of a tightly controlled complex bacteriocin expression system, the pediocin PA-1 genetic organization features the other extreme, with a basic organization of a single gene cluster composed of only four genes pedABCD expressed in a single operon. Pediocin PA-1 production is ensured by the pedA, the pediocin precursor gene and pedCD encoding a dedicated ABC-transporter, while immunity is encoded by pedB (Rodriguez *et al.* 2002). Additional immunity systems as well as a quorum sensing system are absent. Interestingly, the cluster encoding pediocin PA-1 and highly similar bacteriocins is located on a plasmid that can be and likely has been transferred between species (Cui *et al.* 2012).

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### Lactococcin G

Lactococcins belonging also to the class II bacteriocins (nonlantibiotics). They are mainly active against other *lactococci* and they damage target cells by pore formation or by interfering with the integrity of the membrane (Nes *et al.* 1996). Of the two-peptide bacteriocins (class IIb), lactococcin G (LcnG) is the most studied in relation to its mode of action. It is constituted of the peptides LcnG-$\alpha$ (39 residues) and LcnG-$\beta$ (35 residues) (Rogne *et al.* 2008), and its bactericidal activity relies on causing leakage of $Na^+$ and $K^+$ ions from the membrane of sensitive cells (Moll *et al.* 1996, 1998). The main target of lactococcin G is the membrane protein UppP/BacA, involved in the synthesis of peptidoglycan in the strains *L. lactis* ssp. *lactis* IL1403 and *L. lactis* ssp. *cremoris* MG1363 (Kjos *et al.* 2014).

Lactococcin G cluster is composed of two structural genes encoding the pre-bacteriocins (lagA and lagB), an immunity gene (lagC), an ABC transporter gene (lagD) and, located downstream of lagD, a gene coding for a transport accessory protein (lagE; Oppergard *et al.* 2010). The mechanism responsible for the secretion of lactococcin G by LagD and its dependency on the LagE transport accessory protein are not yet clarified.

*Lactococcus lactis* strains LMG2081 and BGBM50 are known as lactococcin G producers (Niessen *et al.* 1992; Mirkovic *et al.* 2015). The complete sequence of lactococcin G operon (~4.9 kb) in *L. lactis* LMGT 2081 has been deposited in the NCBI database (GenBank accession number FJ938036). BLAST alignment of the cluster against the database revealed the presence of a nearly complete LcnG operon (94% coverage, 97% identity) also on the chromosome of *L. lactis* strain CBA3619 isolated from kimchi.

## OTHER ANTIBACTERIAL COMPOUNDS

The term bacteriocin-like inhibitory substances (BLIS) is used for presumptive bacteriocins still under investigation until their amino acid structure is identified (Settanni and Corsetti 2008). Other antibacterial substances fit into neither the low molecular weight metabolites, bacteriocins nor BLIS categories. They are not less active and sometimes have a broader spectrum of inhibition. Some of the compounds show limited inhibitory effect alone and are more active together with other substances or in combination with lactic acid (pH) producing food culture(s) (Niku-Paavola *et al.* 1999). This was demonstrated in cheese with a mixture of strains (Settanni *et al.* 2011), where not only antimicrobial activity was measured, but also increased growth of the starter cultures. Examples of such antimicrobial compounds showing a broad-range inhibitory activity include reuterin, a compound produced by *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) that inhibits fungi but also Gram-negative bacteria (Schaefer *et al.* 2010), and indeed, inhibition of *Clostridium difficile* has also been shown (Cleusix *et al.* 2007). A further example is D-3-Phenyl-lactic acid, shown to have inhibitory activity towards various pathogens like *Salmonella enterica*, *L. monocytogenes* and produced by several LAB but also *Geotrichum candidum* (Dieuleveux, Lemarinier and Guéguen 1998, Rodríguez, Martínez and Kok 2012).

### Reuterin

As a bacterium occurring in sourdough, dairy and meat products, *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) has gained interest as a potential bioprotective food culture due to its ability to synthesize reuterin, a broad-spectrum antimicrobial system consisting of an isomeric mixture of 3-hydroxypropionaldehyde (3-HPA; Vollenweider *et al.* 2003). Reuterin displays inhibitory activity against bacteria, yeast, moulds and protozoa, including food spoilage and pathogenic organisms (Schaefer *et al.* 2010). Notably, metabolism of glycerol has been demonstrated to improve the competitiveness of *L. reuteri* in sourdough (Lin and Gänzle 2014). *Limosilactobacillus reuteri* uses a CoA-dependent pathway, in which 3-HPA is obtained from glycerol in a reaction catalysed by the coenzyme B12-dependent glycerol/diol dehydratase (GDH; Talarico and Dobrogosz 1990); 3-HPA is subsequently converted to 3-hydroxypropionic acid (3-HP) and 1,3-propanediol (1,3-PDO) (Dishisha *et al.* 2014). The glycerol/diol dehydratase of *L. reuteri* has been shown to be encoded by three genes located in the propanediol-utilization (pdu) operon (PduCDE; Morita *et al.* 2008); adjacent to this operon, *L. reuteri* possesses cbi–hem–cob genes that encode the proteins for the biosynthesis of vitamin B12 (Santos *et al.* 2008). In addition, Srimulu *et al.* (2008) found that glycerol/diol dehydratase is associated with microcompartments called metabolosomes, and their structural proteins are encoded by genes located in the pdu operon. The structure of the pdu-cbi-cob-hem cluster in *L. reuteri*, displaying a putative transposase gene between the pdu and cbi-cob-hem operons and IS elements within flanking regions, suggests this gene cluster may be a genomic island that has been acquired through horizontal gene transfer (Morita *et al.* 2008). Based on this hypothesis, it seems reasonable to assume that other species of LAB besides *L. reuteri* may have acquired this genetic island during evolution. Indeed, glycerol metabolism leading to 3-HPA production has been reported in *Secundilactobacillus collinoides* (formerly *Lactobacillus collinoides*; Sauvageot *et al.* 2000), *Loigolactobacillus coryniformis* (formerly *Lactobacillus coryniformis*) isolated from cheese (Martin *et al.* 2005), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) and *Lentilactobacillus buchneri* (formerly *Lactobacillus buchneri*; Schutz and Radler 1984), *Lentilactobacillus diolivorans* (formerly *Lactobacillus diolivorans*) from ciders (Garai-Ibabe *et al.* 2008). Consistent with this, the pdu operon has been detected in *L. collinoides* (Sauvageot *et al.* 2002) and in *L. brevis* (Makarova *et al.* 2006).

### Phenyllactic acid

3-phenyllactic acid (2-hydroxy-3-phenylpropanoic acid, PLA) is acknowledged as a relevant contributor to the anti-microbial activity of LAB in fermented foods. A wide range of LAB genera, such as *Lactobacillus*, *Leuconostoc*, *Weissella*, *Pediococcus* and *Enterococcus* (Magnusson *et al.* 2003; Valerio *et al.* 2004; Ndagano *et al.* 2011; Li *et al.* 2014), have been demonstrated to produce PLA, though PLA biosynthesis has been most extensively studied in *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*; Lavermicocca *et al.* 2000; Ström *et al.* 2002; Prema *et al.* 2010; Wu *et al.* 2020). Remarkably, PLA exerts inhibitory effects *in vitro* and *in vivo* on several spoilage and mycotoxigenic moulds from sourdoughs and bakery products (Lavermicocca, Valerio and Visconti 2003; Dal Bello *et al.* 2007; Ryan *et al.* 2011; Valerio *et al.* 2016).

In LAB, PLA is a by-product of phenylalanine (Phe) catabolism: phenylalanine is firstly transaminated to phenylpyruvic acid (PPA) by an aromatic aminotransferase (AAT; Yvon *et al.* 1997) and subsequently reduced to PLA by a 2-hydroxyacid dehydrogenase (2-HADH) such as lactate dehydrogenase (LDH; Vermeulen, Ganzle and Vogel 2006; Li, Jiang and Pan 2007; Mu *et al.* 2010). Although the genes encoding such enzymes are ubiquitously present in LAB, significant differences are recorded in the amount of PLA produced by different strains, and this disparity was ascribed to varying enzymatic

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activity of LDH toward PPA (Li *et al.* 2008). Recently, it has been demonstrated that *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) LY-78 synthesizes PLA de novo via the Phe synthetic pathway and suggesting that panE1 (ketopantoate reductase), serA1 (D-3-phosphoglycerate dehydrogenase) and ldhD2 (D-lactate dehydrogenase 2) may be key genes of PLA biosynthesis in LAB (Sun *et al.* 2019).

## CONTROL OF ACIDITY OF FERMENTED FOOD PRODUCTS AS A RESULT OF FERMENTATION

### Acidification mechanism

Many parameters govern the survival and growth of microorganisms in food. The acidity or pH of a food can affect the type and number of microorganisms present. All microorganisms have an optimum pH value for growth, and altering the hydrogen ion concentration can influence the growth of an organism or even inhibit growth. In general, bacteria prefer to grow at a pH near neutrality (pH 6.5–7.5) but will tolerate a pH range of 4–9. Yeasts are more tolerant of lower pH values than bacteria, while moulds survive across the widest range of pH values. Foods with a pH value below 3.5 can support the growth of both yeasts and moulds. Because of the sensitivity of organisms to widely differing pH values, the pH provides a powerful selection which influences the species or group of microorganisms that will predominate in unaltered food products. For example, bacteria primarily spoil proteinaceous foods such as dairy, meat, poultry and seafood with a pH range of 5.5–6.5. In contrast, yeasts and moulds more commonly proliferate on fruits and vegetables with inherently lower pH values and little buffering capacity (Doores 2005).

One effective means to preserve food from spoilage is to increase the acidity of the food thereby creating an unfavourable environment for survival of undesirable microorganisms through natural fermentation. Depending on the final pH, this inhibition can be either biostatic or biocidal. The final result will depend on the microbial species, the type and concentration of the acidulant, the time of exposure, the buffering capacity of the food and most likely the compositional/chemical properties of the food (Doores 2005).

Microorganisms display varied sensitivity to acids. In most cases, they are vulnerable to the organic acids they produce because they are by-products of primary metabolism and as such are their natural 'electron sink'. It may happen, however, that in mixed culture fermentations, the produced acid is a source of energy for another microorganism. For example, LAB produce lactic acid as a by-product of their metabolism, which serves as an energy source for propionic acid bacteria (PAB) and the mould *Penicillium roqueforti*. *St. thermophilus* produces formic acid as a by-product of their metabolism, which serves as an energy source for *L. bulgaricus* in yogurt fermentation.

Organic acids are weak acids, so they do not dissociate completely in aqueous environments, and their antimicrobial activity depends on the degree of dissociation and the pH in the food environment. Hence, the antibacterial activity of organic acids is increased when the pH of the food is low. A decrease in pH leads to an increase in protonated acid concentration, decreasing the polarity of the molecules and consequently increasing the diffusion of acids across the cell membrane and into the cytoplasm (Doores 2005; Mani-López, García and López-Malo 2012). The inhibitory effect of organic acids is based on pH, concentration, chain length, type and degree of branching. Indeed, effective use of an acidulant depends on the dissociation constant (pKa) or the pH at which 50% of the total acid is dissociated. The pKa of most organic acids is between pH 3 and 5. The pKa of the acids most commonly found in fermented foods are: acetic acid, pKa 4.75; lactic acid, 3.08 and propionic acid, 4.87. Because the undissociated portion of the molecule is believed to be responsible for the antimicrobial effect, it would be advantageous to use the acids near these values from a biopreservation perspective. As the pH of a solution decreases, the concentration of the undissociated form will increase for all acids. For weaker acids, the undissociated proportion at any given pH will be higher than for stronger acids, i.e. those with a lower pKa (Adams 2014).

In foods having a range of buffering components such as proteins and amino acids, it is not easy (possible) to calculate a degree of dissociation of weak acid using the Henderson–Hasselbach equation. The issue may be further complicated by the presence of oil or fat into which the acid might partition preferentially. This would have the effect of decreasing the acidity in the aqueous phase in which microbial growth occurs and thereby the anticipated antimicrobial effect (Wilson, Wilson and Waspe 2000).

Because pH values below 4.5 will stop or severely curtail the growth of all the major bacterial pathogens and will, depending on the conditions, ultimately lead to their death/inactivation, food safety concerns tend to be much reduced when considering acidic/fermented foods. It has long been recognized that *Clostridium botulinum* spores will not germinate and grow at pH values below 4.6, and this is enshrined in various codes of practice. Usually, this organism can be well controlled by efficient acid production. Levels of acidity which do not kill pathogens or stop their growth entirely can still improve food safety. The risk from infectious pathogens such as *Salmonella* spp. will be lower if growth and thereby numbers of the organisms are restricted and, at suboptimal pH, toxigenic organisms such as *Staphylococcus aureus* cannot grow to levels sufficient to produce biologically effective concentrations of toxin in the food. This is exemplified in EU regulations where food safety criteria for milk powder and some cheeses specify that only when levels of coagulase positive *staphylococci* exceed $10^5$ CFU/g, there is a requirement to test for enterotoxin content (Adams 2014).

### Lactic acid fermentation

Lactic acid is produced by many microorganisms of which the most known are LAB belonging to genera former *Lactobacillus* genus, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Streptococcus* (Crowley, Mahony and van Sinderen 2013).

There are three different pathways leading to production of lactic acid from glucose (lactose); substrate level phosphorylation (homofermentative process) leading to production of two molecules of lactic acid from one of glucose; 6P-Gluconate pathway (heterofermentative process) leading to production of one molecule of lactate, ethanol and carbon dioxide from one molecule of glucose; and the Bifidus pathway leading to production of two molecules of lactate and three acetate from two molecules of glucose (Kandler 1983). The produced acid is in the form of L(+), D(-) or as racemic lactic acid D(-) and L(+). The production and secretion of lactic acid and other weak organic acids results in an acidic environment which generally restricts growth of both bacteria and fungi, including many pathogenic and spoilage microbes (Ross, Morgan and Hill 2002). Acid production and a simultaneous reduction in pH are inevitable consequences of LAB growth, and acidity levels in some fermentations can exceed 100 mM, reducing the pH to below 4.0 in weakly buffered systems (Adams and Mitchell 2002).

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The rate of pH drops and the final pH value in lactic acid fermentations depend on a number of factors such as the buffering capacity and water activity of the medium, the temperature and duration of fermentation, the inoculum size and the metabolic activity of the bacteria. Ideally, the target pH would be around 4.5, although this is not achieved in many common fermented foods such as cheese. Even in very weakly buffered media, the pH would tend to level off around 3.8 as lactic acid production produces a lactate buffer. Maximum effect will also be achieved if the pH drop occurs rapidly, within hours, to prevent any pathogen growth occurring (Adams 2014).

### Acetic acid fermentation

The microorganisms oxidizing ethanol to acetic acid are commonly called acetic acid bacteria (AAB). Acetic acid bacteria are mesophilic obligate aerobes that oxidize sugars, sugar alcohols and ethanol, with the production of acetic acid as the major end-product. During acetic acid production, ethanol is almost quantitatively oxidized to acetic acid. Acetic acid bacteria exhibit resistance to high acetic acid concentrations and low pH (Raspor and Goranovič 2008; Yamada and Yukphan 2008). Physiologically, bacteria belonging to the genus *Acetobacter* sp. convert alcohols to acids by oxidation.

### Propionic acid fermentation

Propionic acid bacteria (PAB) are combined into the family *Propionibacteriaceae*, genus *Propionibacterium* and *Acidipropionibacterium*. They are isolated from milk, fermented dairy products and cheese; they are also found in at least 24 different vegetables and fruits species (Vorobjeva, Khodjaev and Vorobjeva 2008).

Depending on the strains, the ratio of PA to AA can vary widely and well beyond theoretical 3:1. Their product, PA alone or with AA, is inhibitory toward *Aspergillus flavus*, aerobic *Bacillus*, *Salmonella* spp. and yeasts and has been used as a mould inhibitor for animal feed, wet corn, silage and grain (Balamurugan, Venkata and Panda 1999) as well as in the food industry to prevent spoilage of foods such as bread and cake from moulding.

## ECOLOGICAL COMPETITION

Microorganisms inhabit almost every environment in the world, including different food matrices. Interactions between different microorganisms are unavoidable and can either be symbiotic or competitive. Mechanisms of interaction include e.g. the production of inhibitory molecules and the competition for nutrients. Andreevskaya *et al.* (2018) showed that *Leuconostoc gelidum*, a spoilage LAB, in packaged cold-stored meat products enhanced its nutrient-scavenging capabilities in the presence of *Lactococcus piscium* and *Paucilactobacillus oligofermentans* by upregulation of carbohydrate catabolic pathways, pyruvate fermentation enzymes and ribosomal proteins. The slower growing *Lc. piscium* and *P. oligofermentans* downregulated these functions in the presence of *Le. gelidum*, but overexpressed prophage genes and restriction modification systems, which are mechanisms of DNA exchange and protection against it (Andreevskaya *et al.* 2018). Several studies on growth competition of spoilage organisms and potential bioprotective strains in media or food models exist. The mechanism of action responsible for growth inhibition is unfortunately often not examined. As an example: Leyva Salas *et al.* (2018) tested the antifungal activity of 32 LAB strains against the four fungi *Penicillium commune*, *Mucor racemosus*, *Galactomyces geotrichum* and *Yarrowia lipolytica* to find a combination of food cultures, which has a bioprotective effect without influencing the organoleptic properties nor inhibiting starter cultures of the respective foods. They used a cheese and yogurt model system and received different outcomes for the respective cultures in the different models, showing that food matrix and production conditions play a significant role in determining antifungal activity of potential bioprotective food cultures.

A way for microorganisms to colonize a favourable environment is either to colonize this environment as soon as it is available, e.g. through fast growth, or to actively displace already existing microorganisms, e.g. by production of antimicrobial compounds (Hibbing *et al.* 2010). When considering antifungal activity of LAB, many studies have revealed that antifungal compounds are often found in concentrations below the minimal inhibitory concentration (MIC), thus pointing out that other mechanisms must be responsible for the bioprotective effects of LAB against yeasts and moulds as well (Siedler, Balti and Neves 2019). The competition for limited resources, for example nutrients, carbon source and essential ions, can result in inhibition or delay of growth of either protective cultures or spoilage organisms. Honoré *et al.* (2016) found that the inhibition of *Penicillium* spp. by *Lacticaseibacillus. paracasei* (formerly *Lactobacillus paracasei*) in a defined medium was not only induced by metabolite formation, but also by nutrient consumption, especially by the consumption of glucose and glutamine. Reduced glucose availability induced by *Penicillium chrysogenum* growth was also assumed to be the reason for repressed Ochratoxin A (OTA) production of *Penicillium nordicum* (Delgado *et al.* 2019).

Furthermore, nitrogen is a limiting factor for microbial growth in dairy-based systems. Micro-organisms initially compete for free amino acids and small peptides, while they compete for peptides in later stages of fermentation. The ability to utilize amino acids efficiently is essential for growth of the respective microorganism (reviewed in Sieuwerts *et al.* 2008). Transcriptome analysis was used to study the interactions of *Lc. lactis* and *Saccharomyces cerevisiae* in coculture during the exponential growth phase. Whereas the lactic acid concentration was the same in a single culture of *Lc. lactis* and in the mixed culture of *Lc. lactis* and *S. cerevisiae*, ethanol concentration and glucose consumption were increased in the mixed culture. Also, pyrimidine metabolism of *Lc. lactis* was reoriented, most likely regulated by the ethanol production of the yeast (Maligoy *et al.* 2008).

Manganese was found to be the limiting factor for dairy yeast and mould growth in yogurt with *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*) and *Lb. paracasei* as protective food cultures (Siedler *et al.* 2020). The manganese transporter (MntH1) is responsible for manganese uptake under acidic conditions and gives strains expressing the mntH1 gene the ability to take up manganese. The resulting manganese depletion ends up in delayed yeast and mould growth. Homologues of the mntH1 gene were found in 15 different *Lactobacillus* species, indicating that this mechanism of competitive exclusion could be a general mechanism of LAB to interfere with yeast and mould growth (Siedler *et al.* 2020).

Iron is another nutrient which is fundamental to bacterial growth and thus a benefit for some micro-organisms if they are able to produce iron scavenging molecules, so called siderophores, to acquire iron from the environment (Hibbing *et al.* 2010). This has been shown for ripening bacteria on the surface of smear-ripened cheese. The addition of either iron or the

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siderophore desferrioxamine B stimulated growth of *Arthrobacter* spp., *Corynebacterium* spp. and *Brevibacterium* spp. Furthermore, genes for iron-siderophore transporter binding proteins of *Arthrobacter arilaitensis* were upregulated when the siderophore was added, but not when iron was added to the medium (Monnet, Back and Irlinger 2012). Sipiczki (2006) hypothesized that contrary to bacterial siderophore production, *Metschnikowia* spp. inhibits growth of other micro-organisms by immobilizing iron in the medium due to formation of an insoluble pigment. Additionally, iron depletion by the biocontrol yeast *Metchnikowia pulcherrima* was successfully used to control the postharvest apple-pathogens Botrytis cinereal and Alternaria alternata during apple storage (Saravanakumar *et al.* 2008).

As soon as a microorganism encounters a favourable environment, binding and attachment are crucial to colonize the eological niche. Many pathogenic bacteria are secondary colonizers of biofilms (Giaouris *et al.* 2015). Shaping the biofilm present on food (e.g. smear cheese) or processing environment (e.g. stainless steel) through the application of protective cultures is a challenging but promising approach. As an example: Habimana *et al.* (2009) studied the attachment of *L. monocytogenes* on biofilms formed by different customized *L. lactis* strains and found that the adhesion of planktonic cells was almost prevented when exopolysaccharides were formed by the biofilm-forming cells, but the adhesion was increased when biofilms had a porous structure formed by chain-making strains.

## FOOD CULTURES AND BIOPRESERVATION: INDUSTRIAL APPLICATIONS

In ancient times, food fermentation was a spontaneous event whose outcome was uncertain in terms of quality and safety. Nevertheless, it was a first step for improving the shelf life (Farnworth 2008). Through the centuries, the monitoring of this process regularly improved, from empirically developed good practices in households to a systematic documentation of HACCP procedures in modern food processing industry. Next to pasteurization, salting and other hurdles, the application of food cultures in traditional fermented foods such as yoghurt, cheese, fermented meat, vegetables, beer and wine is nowadays strongly established. There is also a trend towards the use of traditional biotechnology for developing new foods and beverages (Laranjo, Potes and Elias 2019). Food cultures produce a high variety of compounds, including organic acids, alcohol and aroma compounds, contributing to product texture, taste and safety. This biopreservation contribution to safety meets the growing demand of the consumers for minimally processed food products.

The application of food cultures, which is widespread in dairy, meat and vegetable products, is already a form of biopreservation, but the addition of protective food cultures with a better productive effect can add another hurdle against pathogens or spoilage organisms. This is especially the case for fresh and RTE food products which lack a heating treatment and contain therefore potential spoilage organisms.

Applied research in the area of protective effect of food cultures is a challenging task. Between the discovery of an inhibitory activity in synthetic media and a commercially available food culture, a protective food culture has to pass the so-called challenge tests. It is thereby inoculated in a given food matrix together with the target microorganism and evaluated for the ability to reduce or control the outgrowth of the target organism over the desired shelf life. Applied research has been carried out extensively in the last two decades focusing on inhibition of pathogenic microorganisms such as *L. monocytogenes*, *Salmonella* sp., Shigatoxin producing *Escherichia coli* and *Staphylococcus aureus* as well as spoilage microorganisms such as yeasts, moulds and *Clostridium* species (Settani *et al.* 2008; Castellano *et al.* 2017; Leyva Salas *et al.* 2017; Bosse Née Danz *et al.* 2018; Oliveira *et al.* 2018; Silva, Silva and Ribeiro 2018; Laranjo, Potes and Elias 2019; Rouse and van Sinderen 2008). Well-adapted microorganisms will provide a more efficient protection with more effective inhibitory microorganisms often being isolated from the investigated food itself (Austin-Watson, Grant and Brice 2013; Lee *et al.* 2016; Scatasa *et al.* 2017). However, care must be taken that those cultures do not have a negative impact on the organoleptic characteristics of the end-product.

Studies covering a wide range of foods including all fermented foods listed above as well as non-fermented foods are presented below.

### Dairy products

As first line of defence, fermented dairy products are preserved through the acidification carried out by food cultures, as illustrated by the high safety of yoghurt. Thanks to its low pH, yoghurt is only susceptible to yeast and moulds (Leyva Sala *et al.* 2017), but recently a general mechanism of LAB to also inhibit yeast and mould growth was discovered (Siedler *et al.* 2020). The careful selection of strains constituting mesophilic food culture of mildly acidified products such as cheese can already substantially increase the protection against pathogens and spoilage agents, as extensively shown for the species *L. lactis* (Silva, Silva and Ribeiro 2018). The applications mainly cover the protection against *L. monocytogenes* and *Clostridium tyrobutyricum* (Garde *et al.* 2011; Kondrotiene *et al.* 2018; Lianou and Samelis 2014; Samelis and Kakouri 2018). *Lactobacillus* spp. and *Enterococcus* spp. as an adjunct culture may also increase food safety (Cocolin *et al.* 2007; Martinez *et al.* 2015). Raw milk soft cheeses are highly sensitive to contamination by gram-negative bacteria such as *Salmonella* spp. and Shigatoxin producing *E. coli*. Application of LAB together with the gram-negative species *Hafnia alvei* was shown to protect those cheese types (Callon, Arliguie and Montel 2016). Non-fermented dairy products such as cottage cheese also benefit from the addition of selected protective food cultures as shown by numerous studies (Silva, Silva and Ribeiro 2018; Chhetri, Prakitchaiwattana and Settachaimongkon 2019).

By analogy to bioprotection observed in traditional fermented food, a combination of strains is often more powerful than a single strain (Aljasir *et al.* 2020; Aljasir and D'Amico 2020; Rodriguez *et al.* 2012; Chhetri, Prakitchaiwattana and Settachaimongkon 2019; Sindi *et al.* 2020).

While research mainly focuses on LAB, further microorganisms isolated from the food microbiota may also contribute to food safety. One research field focuses on the rind of ripening cheeses that may support survival of pathogens (Roth *et al.* 2011; Imran *et al.* 2013; Callon *et al.* 2014). As the biodiversity of rind microbiota is by far higher than the core microbiota, the development of future protective food cultures relies on a deep understanding of the species interactions in this habitat. In many cases, such added food cultures produce inhibitory compounds against various pathogens or spoilage bacteria, but in certain cases a combined effect of the starter together with the protective food culture inhibits growth. In many cases, *L. lactis* subsp. *lactis* is involved either as protective food culture or as starter culture (Kondrotiene *et al.* 2018), but other species

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such as *Latilactobacillus sakei* (formerly *Lactobacillus sakei*) or *Enterococcus* spp. can also be used and this demonstrates that no regulatory difference should be made (Cocolin *et al.* 2007; Martinez *et al.* 2015). Another application is the use against *Staphylococcus aureus* (Aljasir and D'Amico 2020). In certain cheeses such as cottage cheese, halophilic food cultures can be used also against *S. aureus* (Chhetri, Prakitchaiwattana and Settachaimongkon 2019).

The prevention of growth of *Salmonella* spp. in dairy products can also be achieved using specific cultures added to dairy products. *Salmonella* spp. was inhibited in cheese using a *Hafnia alvei* food culture, and the same authors showed the increased inhibitory effect of the combination of selected food cultures against *L. monocytogenes* (Callon, Arliguie and Montel 2016; Aljasir *et al.* 2020).

A combination of several species/strains for the inhibition of pathogens, which was usually the case in the early days of fermentation, can enhance the antimicrobial activity due to either increased inhibitory power of a wider range of target organisms. This is, for example, the case for kefir products which has been described to have antimicrobial properties attributed to its low pH and specific antimicrobial substances produced during the fermentation process (Kim *et al.* 2016; Sindi *et al.* 2020).

### Meat and fish products

There are various hurdles used to preserve meat and fish products, including addition of salt, nitrite, starter cultures, smoking and cold storage. There is increasing evidence that protective food cultures may also play a significant role against *L. monocytogenes*, toxin-producing *Staphylococcus aureus* and further pathogenic and spoilage microorganisms (Castellano *et al.* 2017; Bosse Née Danz *et al.* 2018; Oliveira *et al.* 2018; Laranjo, Potes and Elias 2019; Aljasir *et al.* 2020). In meat, nitrite is a salt with potential toxic effects, and there is pressure to reduce its use. A combination of nitrite with a protective food culture demonstrated a higher reduction of *L. monocytogenes* in fermented sausages than without the addition of the protective food culture (Nikodinoska *et al.* 2019). The combined effect of food cultures has been shown to inhibit Ochratoxin A production in dry cured ham by *Penicillium chrysogenum* and *Debaryomyces hansenii* (Cebrian *et al.* 2019).

Since fish and meat are not sterile (Just like RTE Food products), their own microbiota will be active during storage. Psychrophilic microbiota already present on meat and fish such as *Carnobacterium* spp. and *Lactococcus piscium* can inhibit growth of pathogenic and spoilage bacteria. This is very promising, but careful selection of strains has to be carried out, as those highly adapted species will either spoil or protect the product, depending on their strain-specific phenotypes. (Castellano *et al.* 2017; Zhang, Gänzle and Yang 2019; Bazarnova *et al.* 2020)

### Vegetables and cereal products

Moulds are the major spoilage issue when it comes to preservation of vegetables and cereal products. For a detailed overview of antifungal protective cultures in this type of food, we suggest reading the following recent review (Leyva Salas *et al.* 2017).

*Listeria monocytogenes* is also a pathogen of concern for vegetables. The application of protective cultures can be either directly on the product or through the washing process. Ramos *et al.* (2020) showed that a *Pediococcus pentosaceus*, prevented *Listeria* sp. proliferation in vegetable. In cabbage, the application of a *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) strain reduced *L. monocytogenes* but showed at the same time that it is also important to synchronize all hurdles since the absence of oxygen increased the resistance of *L. monocytogenes* against *L. plantarum* (Dong *et al.* 2020).

Sourdough bread is a fermented product using yeasts and LAB. This microbiota can naturally protect the product from spoilage such as moulds (Chavan and Chavan 2011). In the case where the fermentation process for bread is mainly dominated by yeast, the addition of specific LAB food cultures enables the inhibition of for example *Aspergillus* spp., *Penicillium* spp. and *Fusarium culmorum* (Russo *et al.* 2017), thereby increasing safety (reduction of mycotoxins) and quality (reduction of off-flavour).

### Industrial protective food cultures

Thanks to extensive scientific investigation in the last decades, food producers start to have access to protective cultures specifically developed to control a given pathogenic or spoilage microorganism in a given food. Compared to the tremendous number of studies dedicated to the development of protective food cultures illustrated above, there are still only a few available on the market. One cause may be that the protective food culture has to fit into an already intricate hurdle concept. Therefore, models used in future investigations should be as close as possible to the real processing conditions. In particular, care must be taken for commercial bioprotective food cultures not to have a negative impact on the organoleptic characteristics of the product. The regulation of use and declaration of protective cultures nevertheless remains an intricate topic for regulators in some regions/countries. The protective and preservation effects are seen as a key new effect of food cultures and it is regarded as belonging to food additive.

## SAFETY DEMONSTRATION OF FOOD CULTURES FOR THEIR BIOPROTECTION ACTIVITIES

There is currently no firmly established regulation for the safety assessment of live micro-organisms added to food products as cultures or ingredients. However, there are a number of guidelines, recommendations and expert reviews on possible steps to document and validate the safety of live microorganisms used in foods independent of the mode of action of the food cultures (Laulund *et al.* 2017). Most industrial food strains used today are bacteria from species with a history of use in food products without apparent adverse effects. Four types of investigations have been proposed as further detailed.

### Opportunistic infections

Commensal bacteria have been described to cause infections in patients with underlying disease (Berg and Garlington 1979; Berg 1985,1995). Owing to its natural presence in different sites of the human body and in fermented food products, the genus *Lactobacillus* has gained particular attention. *Lactobacillus* infections occur at a very low rate in the generally healthy population – estimated 0.5/1 million per year (Borriello *et al.* 2003; Bernardeau, Guguen and Vernoux 2006). As stated in two reviews of *Lactobacillus* infections: 'Underlying disease or immunosuppression are common features in these cases, whereas infection in previously healthy humans is extremely rare' (Aguirre and Collins 1993), and '*Lactobacillus* bacteraemia is rarely fatal per se but serves as an important marker of serious underlying disease' (Husni

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*et al.* 1997) sporadic infections have been reported in immunocompromised patients. The underlying problems have mainly been central venous catheter (CVC) in place, metabolic disorders, organ failure, or invasive procedures as dental work (Axelrod *et al.* 1973; Liong 2008). Infections by other bacterial species used as food cultures are also extremely rare (Horowitz *et al.* 1987; Barton, Rider and Coen 2001; Mofredj, Bahloul and Chanut 2007; Leuschner *et al.* 2010).

Infections with the commonly used yeast and mould species are rare events as well (Enache-Angoulvant and Hennequin 2005). Most of the infections are due to opportunistic pathogens not recognized as food cultures and affect immunocompromised patients and hospitalized patients (Jacques and Casaregola 2008; Miceli, Diaz and Lee 2011). In the 2018 re-evaluation EFSA concluded: 'The safety concerns described are all considered linked to severe underlying health conditions and therefore do not change the consideration of Lactobacillus spp. for the QPS status' (EFSA 2018).

### Toxic metabolites and virulence factors

Biogenic amine formation in fermented foods by LAB has recently been reviewed (Spano *et al.* 2010). Following food poisoning outbreaks (Sumner *et al.* 1985), metabolic pathways have been elucidated (Straub *et al.* 1995) and screening procedures proposed to limit the level of production (Bover-Cid and Holzapfel 1999, Bover-Cid, Izquierdo-Pulido and Vidal-Carou 2000).

The presence of mycotoxin genes also raises safety concerns, although the level of expression within fermented food is very unlikely to cause any health hazard (Barbesgaard, Heldt-Hansen and Diderichsen 1992). Within fungi, the potential for antibiotic production is also an undesired property.

The occurrence of virulence traits should not be present in microorganisms used in a food fermentation. A specific risk assessment should be conducted on strains presenting these undesirable properties, even if they belong to a species with a long history of use (Semedo *et al.* 2003a, b).

### Antibiotic resistance

The emergence and spread of antibiotic resistance are a major global health concern. The on-going Codex ad hoc intergovernmental task force on antimicrobial resistance is focused on the non-human use of antimicrobials. Microorganisms intentionally added to food and feed for technological purposes have not been shown to aggravate the problem of spreading antibiotic resistant pathogens (Anonymous 2011).

Intrinsic resistance or resistance that is caused by mutation in an indigenous gene not associated with mobile elements would represent a very low risk of dissemination (Saarela *et al.* 2007). Acquired antibiotic resistance genes, especially when associated with mobile genetic elements (plasmids, transposons), can be transferred to pathogens or other commensals along the food chain from within the product until consumption (FEEDAP 2005, 2008; Nawaz *et al.* 2011).

The role of microorganisms in the spread of antibiotic resistance has been assessed in fermented foods (Nawaz *et al.* 2011). Results of such studies confirm the role of a reservoir of antibiotic resistance genes from the food microbiota, without identifying any major health concerns to date.

It is considered that strains carrying acquired antibiotic resistance genes might act as a reservoir of transmissible antimicrobial resistance determinants (FEEDAP 2005, 2008). Gene transfer of antibiotic resistance between microorganisms in the food and feed chain is thus considered to be a topic of surveillance for the safety demonstration of microorganisms (Borriello *et al.* 2003; Gueimonde *et al.* 2005).

### Definition of 'History of use'

The concept of 'history of safe use' has appeared recently in regulations and in safety assessment guidance. One definition of 'history of safe use' proposes ''significant human consumption of food over several generations and in a large, genetically diverse population for which there exist adequate toxicological and allergenicity data to provide reasonable certainty that no harm will result from consumption of the food'' (Health Canada 2003). In order to evaluate the history of safe use of a microorganism, it is necessary to document not just the occurrence of a microorganism in a fermented food product, but also to provide evidence of whether the presence of the microorganism is beneficial, fortuitous, or undesired.

## CONCLUSION

Biopreservation of a food product can be achieved by fermentation, in a targeted or untargeted way. While the different bacteriocins described (mostly from LAB) show a dedicated mechanism to ensure primarily the survival in an ecosystem of a microbial species, but serve also as an additional hurdle to ensure safe food, other metabolic pathways, such as acidification, most specifically preserve initially short life stable food matrices (uninoculated milk get spoiled within hours). The initial empiric use of fermentation in the food chain was done for extended shelf life and avoiding food waste and food lost. Adaptation to texture and sensorial properties came after. The consumer has adapted its taste preferences to the different food cultures, and what is fermented food for one population and part of its culture can be considered spoiled if not dangerous by others.

Biopreservation itself can be ensured by other biological mechanisms than fermentation that were not considered in the present review. They would require a different approach, both for usage and safety demonstration. The potential of application of food cultures for biopreservation should not be considered something 'new' *per se* requiring a specific regulation and application process. It remains a traditional use of food cultures in the food chain, most presumably its first use. The focus in the initial steps of food microbiology on the sensorial properties has had a counterproductive effect on the application of food cultures for other applications. Fermentation applied to biopreservation should not be understood as a new approach, but one that time made us forget. Its potential as an additional hurdle to tackle food waste in combination with the numerous control measures already in place should not be underestimated. Enabling the delivering of safe, stable and tasty foods fitting in a sustainable lifestyle.

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Food Cultures to address eventual concern regarding the safe use of microorganisms in fermented foods.

*Conflicts of Interest.* None declared.

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