Food ingredients synthesised by lactic acid bacteria

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1. Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of strains of different genera belonging to the *Lactobacillales* and *Bifidobacterium* orders, which synthesise lactic acid as the major product of sugar fermentations. They are widely known for their industrial applications mainly as starters for food fermentation, as biocontrol agents and as probiotics. They have a "generally recognized as safe (GRAS) status" as defined by the US Food and Drug Administration Agency. In addition, LAB constitute an important candidate group for metabolic engineering strategies due to several well characterised properties including the well-understood genetics of *Lactococcus lactis* and *Lactobacilli* species, which facilitates genetic engineering (Gaspar et al., 2013).

LAB can adapt to many environmental conditions in order to survive, since they colonize many food matrices such as milk, meat, vegetables and grains and they are members of the digestive tract and vaginal microbiota of humans and other animals. For this colonization to occur, LAB's complex nutritional requirements must be provided for, including fermentable sugars and also several other essential components, to ensure and/or to improve their metabolism, and thereby produce an important number of metabolites with wide applications in the food and pharmaceutical industries (Mazzoli et al., 2014).

LAB are classified into two groups according to their hexose metabolic pathways: (i) homofermentative (HoLAB), where lactic acid is the main end-product and (ii) heterofermentative (HeLAB), which produce other end-products such as acetic acid, carbon dioxide or aroma compounds, in addition to lactic acid. The latter is one of the many high-value compounds which can be produced by LAB´s metabolisms, which also synthesise biofuels such as ethanol and butanol, polysaccharides, antimicrobial agents, health-promoting substances and nutraceuticals such as vitamins and polyols

(Nuraida, 2015).

These metabolic features constitute LAB's key contribution to fermented food manufacture. The production of lactic acid, which causes decrease of the pH, preserves several foods by inhibiting the proliferation of undesirable microorganisms. This acid also participates in the formation of flavour and in the coagulation of caseins (major milk protein), which modify the texture of products such as cheese and other fermented milk (Papagianni, 2012). The production of carbon dioxide plays an important role for the texture of serval kinds of cheese including the formation of "eyes" in the Swiss-style products such as Emmental. For blue cheese, metabolism of HeLAB (genera *Leuconostoc*) results in the release of carbon dioxide, which forms cavities in the coagulum where colonization by *Penicillium roquefortii* contributes to the final texture and flavour. In addition, carbon dioxide can itself play a role as antimicrobial agent (Saranraj et al., 2013) and LAB also produce several other compounds with antimicrobial (Woraprayote et al., 2016) and antifungal activities (Tropcheva et al., 2014). Among these are organic acids, hydrogen peroxide, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins, which contributes to the conservation and preservation of various food products (Yang et al., 2012). Also, the synthesis by LAB of metabolites such as diacetyl, acetoin, acetaldehyde and α-keto acids contributes to the aroma and flavour of dairy products (Smid and Kleerebezem, 2014). Furthermore, the production of exopolysaccharides (EPS) by LAB improves the viscosity and texture of fermented products, such as yogurt, kefir, cheese and cereal-based products. In addition, EPS have potential health benefits (Pérez-Ramos et al., 2015) and are classified as prebiotics (Monedero et al., 2010). Moreover, the production of polyols by LAB plays a role in diabetic food products as a low caloric food ingredient (Monedero et al., 2010). Finally, the production of vitamins by LAB is also important, due to the

role of these micronutrients in the metabolism of all living organisms (Capozzi et al., 2012).

Consequently, in this chapter we review the current status of the knowledge and usage of various LAB metabolites and the future perspectives in this field.

2. Exopolysaccharides produced by LAB

Some LAB are able to synthesise glycosidic polymers. These compounds are usually secreted (exopolysaccharides, EPS) and remain linked to the cell surface, typically covalently forming a capsule, or loosely bound, or freely released to the environment (Suresh Kumar et al., 2007). So far LAB belonging to the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Oenococcus*, *Streptococcus* and *Weissella* have been identified as EPS producers (Garai-Ibabe et al., 2010, Amari et al., 2013, Rühmkorf et al., 2013).

The various EPS from LAB differ in: (i) Composition; containing different types of monosaccharide units joined by different types of linkages; (ii) structures with different degrees and types of branching; (iii) molecular mass, and (iv) overall, structural conformation.

The roles that EPS can play in food depend on these different characteristics as well as on the concentration of the polymers in the food, and on their interaction with the food matrices. Based on their composition EPS are classified as (i) homopolysaccharides (HoPS), constituted by a single type of monosaccharide and (ii) heteropolysaccharides (HePS) composed of two or more types of monosaccharides. Depending on the linkage type and the orientation of the carbon involved in the bond, HoPS can be classified into four groups: α-D glucans, β-D glucans, β-D fructans and others, such as polygalactans. According to the linkages (shown in brackets) in the main chain, the α-D glucans are subdivided into dextrans (α-1,6), mutans (α-1,3), glucans (α-1,2), reuterans (α-1,4) and

alternans, (α-1,3 and α-1,6). The β-D fructans are subdivided into levans (β-2,6) and inulins (β-2,1) (Pérez-Ramos et al., 2015). The production of β-D-glucans by LAB is limited to a small number of strains associated with the fermentation of alcoholic beverages (Garai-Ibabe et al., 2010). These strains produce a type of β-D glucan with β-(1,3) linkages in the main chain and branches more or less frequently in position β-(1,2). In the case of HePs, there is a backbone of repeating subunits, composed of different monosaccharides (from three to eight), which may be linear or branched and frequently have a range of different linkage patterns. The monosaccharide may be unsubstituted (D-glucose, D-galactose, L-rhamnose, etc.), monosaccharide amines (N-acetylglucosamine, N-acetylgalactosamine, etc.) or monosaccharide substituted with phosphate, acetyl and glyceryl groups, etc (Werning et al., 2012).

Despite the interest in the diversity of EPS produced by LAB, especially because they are GRAS grade additives, they have not yet been used industrially in isolated forms mainly due to the higher prices of bacterial polysaccharides compared with polysaccharides isolated from plant, algae and animal sources (starch, galactomannans, pectin, alginate, etc.). However, the plant and seaweed polysaccharides are not sufficiently reliable, are variable consistency or the quantities available are not sufficient for the market demand (Zannini et al., 2016). Nevertheless, the EPS isolated from LAB have potential industrial applications such as their role as thickeners, stabilizers and emulsifiers as well as gelling, viscosifying and water-binding agents.

Notwithstanding the above, in many industrially produced foods, EPS production by LAB takes place by *in situ* fermentation and plays an important role in their organoleptic properties. Their presence in yogurt, cheese and cereal-based products improves the rheological and organoleptic properties of these fermented products. In addition, some EPS have beneficial health effects such as anti-cholesterolemic and

antitumor effects; they are able to reduce biofilm formation by pathogens (Patel and Prajapati, 2013), and β-glucans produced by LAB have prebiotic (Russo et al., 2012) and immunomodulating activities (Notararigo et al., 2014). Therefore, inclusion of these EPS in food might also contribute to human health. However, prior to that for some usages it will be essential to develop procedures to achieve growth of the producing bacteria in low cost culture media, to improve yield and recovery of EPS from culture supernatants and/or to encourage their production *in situ* (Notararigo et al., 2013).

Currently, it is clear that EPS synthesised by LAB play a role, and have further potential for preparation of dairy and cereal-based products and this subject will be further reviewed in this section (Table 1).

Insert Table 1

2.1. EPS in dairy products. Currently, consumers demand natural and healthy dairy products, creamy, without additives and with low levels of fat and sugar; all these are required without high cost and without altering the general properties of the products (texture, aroma and flavour). EPS are able to increase the viscosity and/or to improve the texture and structure of the food products. Thus, they are contributing to the mouthfeel, without altering the taste and aroma. Although LAB synthesise EPS in small quantities, the amounts are often sufficient to texturize fermented milk products. Moreover, some of them provide an added value, due to the potential beneficial health effects (discussed further, below)

Yogurt is a fermented milk product produced by two LAB: *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus*. These two bacteria have a synergy, because *L. bulgaricus* grows first and produces metabolites that *S. thermophilus* needs for growth. The latter then produces formic acid and carbon dioxide, which further stimulates the growth of *L. bulgaricus* (Badel et al., 2011). *L. bulgaricus* and *S. thermophilus* produce *in situ* HePS (60-150 mg/L and 30-890 mg/L,

respectively), and contribute to the texture of the yogurt. In addition, these bacteria ferment lactose present in milk and produce lactic acid that causes an increase in acidity (to about pH 5.5). This low pH during production of yogurt causes a destabilization of casein micelles, which affects the texture and causes syneresis. Some of the technological approaches used to correct these effects have been to increase the proportion of solid milk proteins, sugars and stabilizers. However, all these measures are not in accordance with the requirements of consumers. The use of starter cultures producing EPS or an adjunct culture, such as the strain *Lactobacillus mocosae* DPC 6426, has been found to improve water retention in yogurts, decreasing syneresis and improving the rheological characteristics (viscosity and elasticity). (London et al., 2015). In addition, results obtained using co-cultures of EPS-producing *S. thermophilus* indicated, that a critical concentration of EPS and other factors must be present to improve yogurt texture (Folkenberg et al., 2006). Moreover, inulin, which is a prebiotic EPS, has been used to produce functional food with improved rheological properties, such as the recent development of new functional sheep milk yogurts (Balthazar et al., 2016).

Kefir is a fermented milk drink, typical of Eastern Europe countries. Kefir grains consist of a polysaccharidic matrix containing LAB (*Lactobacillus acidophilus*, *Lactobacillus kefirgranum*, *Lactobacillus kefir*, and *Lactobacillus parakefir*), yeasts (*Candida kefir*, *Saccharomyces* sp.) and acetic acid bacteria (*Acetobacter* sp.). After use the grains can be dried and reused for further milk fermentations. Kefiran is a HePS composed of D-glucose and D-galactose in equimolar proportions and is produced by *L. kefiranofaciens* (Maeda et al., 2004). This HePS is resistant to enzymatic degradation and improves the viscosity and elasticity of the product. This natural polysaccharide might be employed in the future as an alternative thickening agent in dairy products (Hamet et al., 2015).

Cheese is fermented milk, where LAB play different roles. Some species participate in the fermentation process (*L. lactis*, *Leuconostoc* spp., *S. thermophilus, L. delbrueckii* and *Lactobacillus helveticus*), whereas others are implicated in the maturation phase (*Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Enterococcus durans*, *Enterococcus faecalis* and *Enterococcus faecium*) (Settanni and Moschetti, 2010). The transformation of curd in cheese is performed by microbial proteolysis catalysed by LAB and/or other microorganisms naturally present in milk or added by the cheese-maker. The physical properties of cheese are influenced by initial cheese-milk composition, manufacturing procedures, and maturation conditions. The major factors on the body and texture of cheeses is played by moisture, protein, fat, sodium chloride, milk, salts and pH (Lucey et al., 2003).Cheeses with a high fat content may have negative effects for consumer health, so low-fat products are often presented as an alternative. However, decrease of fat in the cheese can have negative effects on their texture, flavour, and cooking properties. The use of EPS-producing strains in cheese making, can help to improve the texture and taste of the cheeses without the need to add extra additives. The HePS produced by *L. bulgaricus*, *L. helveticus* and *Lactobacillus casei* influence the rheological characteristics of cheese (Broadbent et al., 2001). Moreover, inulin has been used as fat replacer and texturizer for the elaboration of a variety of functional cheeses (Karimi et al., 2015).

EPS, produced *in situ* by LAB, are present in other fermented milks produced in Scandinavian, such as “viili” from Finland, “långfil” and “tatmjolk” from Sweden, “taette” from Norway, “ymer” from Denmark and “skyr” from Iceland. Viili is prepared by fermentation with lactococcal strains (mainly by *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis*). Viili also contains the fungus *Geotrichum candidum*, which causes acidification and a mouldy aroma of the product (Kahala et al., 2008). This fermented

milk is very ropy due to the HePS produced by the bacteria and whose sugar composition is galactose, glucose, and rhamnose (2:2:1). In addition, the EPS acts as a food stabilizer, preventing syneresis and graininess. Besides the effect on food texture, it has been shown that the HePS produced in viili have some health benefits (Ruas-Madiedo et al., 2006).

Based on the above, in the future the *in situ* production of selected EPS produced by LAB should allow the development of new kinds of functional dairy products.

2.2. EPS in cereal-based products. In the baking industry, some EPS can be used to replace or reduce the expensive hydrocolloids (Das et al., 2015), which improve machinability of the dough and rheology as well as the texture, volume and shelf life of bread. Polysaccharide hydrocolloids used in the food industry are mainly extracted from terrestrial plants (e.g. pectin, gum arabic, and hemicellulose) or seaweed (e.g. alginate, agar, and carrageenan). In recent decades, polysaccharides from gram-negative bacteria, such as xanthan synthesised by *Xanthomonas campestis,* have been described as an alternative to plant polysaccharides. The addition of xanthan to a commercial food product must be mentioned in the list of ingredients. The incorporation of additives in bread formulations has several disadvantages, since these products are expensive relative to the price of bread, and do not meet consumers requirements for 'natural' products (Moroni et al., 2009). For this reason, the synthesis of EPS by LAB has gained increasing interest to improve textural properties in bread quality, since this EPS is synthesised *in situ* during fermentation, allowing reduction of costs and avoiding the use of additives. These EPS exhibit a positive effect on the texture, mouth feel, taste perception, and stability (Ruhmkorf et al., 2012), since they influence several technological properties of dough and bread: (i) water absorption of the dough, (ii)

dough rheology and machinability, (iii) dough stability during frozen storage, (iv) loaf volume, (v) bread staling and (vi) crumb structure.

The use of sourdough plays an important role in baking technology by improving aroma, texture, shelf life and nutritional quality (Ganzle, 2014). Sourdough microbiota includes yeast and LAB (Minervini et al., 2014, De Vuyst et al., 2014). The composition of the sourdough microbiota is significantly influenced by endogenous factors in the cereal-based products (carbohydrates, nitrogen sources, minerals, lipids and enzymatic activities) as well as process parameters (temperature, dough yield, oxygen, fermentation time and number of sourdough propagation steps), which result in the selection of specifically adapted LAB and yeast (Ganzle and Ripari, 2016). Acidification, aroma formation and special metabolic activities, like EPS synthesis, are the main function of LAB in the sourdough, which incorporated in bread, improve texture, flavour, volume, shelf life and nutritional value (Ruhmkorf et al., 2012). The LAB strains produce mainly HoPS during sourdough fermentation when sucrose is present (Ruhmkorf et al., 2012). The ability to produce EPS during sourdough fermentation is strain-specific and depends on the metabolic activity of the fermentation microbiota and contributes to the sourdough´s ability to influence bread quality (Galle et al., 2012).

LAB synthesise some HoPS by the action of glycosyl hydrolases, which are able to metabolize sucrose and either use the resulting glucose or fructose or both to synthesise respectively glucans (dextrans), fructans (levans) or alternans. In addition, the metabolism of residual fructose by LAB can results in the formation of mannitol and acetate. The production of acetate is beneficial due to its antifungal properties, but high concentrations may negatively affect taste, dough rheology, bread volume and crumb hardness (Ruhmkorf et al., 2012). Further, apart from dextran synthesis, dextransucrases

can also catalyse sucrose hydrolysis with oligosaccharide formation, and the presence of maltose diverts sucrose conversion from EPS synthesis to the production of prebiotic oligosaccharides (Galle et al., 2010, Galle et al., 2012). Therefore, only those LAB which have the capacity to dominate and compete efficiently with contaminants should be used for specific sourdough fermentation and the final bread quality will depend on this selection (Moroni et al., 2011). *In situ* EPS formation will also be optimized by the selection of the type of flour, dough yield, fermentation time and sucrose concentration (Ruhmkorf et al., 2012).

The use of sourdough has focused on wheat and rye sourdough, where the HoPS formation has been documented for key LAB representatives, including *Lactobacillus sanfranciscensis*, *Lactobacillus reuteri*, *Lactobacillus panis*, *Lactobacillus pontis* and *Lactobacillus frumenti*. In wheat and rye sourdough, levans delay staling, enhance the flavour, and increase the volume of bread (Bounaix et al., 2009).

Currently consumer interest in foods rich in fibre has changed the characteristic of bread products. In these breads, the bran decreases gluten hydration and subsequently disrupts the gluten network formation, which negatively affects the structure of the wheat dough. Dextran production in wheat bran by *Weisella confusa*, acts as a hydrocolloid in this cereal system, improving the quality of high-fibre wheat bread (Kajala et al., 2016, Kajala et al., 2015).

Another current challenge for the baking industry, due to the increasing number of celiac patients, is to develop new baked products for gluten-free diet, since many of the current gluten-free products available on the market are of low quality and particularly exhibit poor mouth feel, dry crumb and flavour (Carini et al., 2015). The production of gluten-free bread is based on the use of gluten-free starch, protein-based ingredients and hydrocolloids, which mimic the viscoelastic properties of gluten (Ruhmkorf et al.,

2012). EPS improve dough rheology and bread texture, and can be used to replace or to reduce hydrocolloids currently used as bread improvers (Moroni et al., 2011, Moroni et al., 2009, Ruhmkorf et al., 2012). A wide range of gluten-free flours is currently available, such as quinoa, sorghum, buckwheat, buckwheat core doughs, rice, and teff. Dextran from *Leuconostoc mesenteroides* and *Weisella* spp. as well as levan from *L. sanfranciscensis* positively affect dough rheology and bread texture in wheat and gluten-free baking (Galle et al., 2010, Katina et al., 2009). The improvement in rheology by dextran producing *Weisella cibaria* and reuteran producing *L. reuteri* in sorghum bread was compared, with dextran production being superior (Galle et al., 2012). Also, HePS produced by *Lactobacillus buchneri* influenced the rheological properties of sorghum sourdoughs but not of wheat sourdoughs (Galle et al., 2011).

There are other cereal-legume-based fermented foods, in which the EPS improves dough rheology. Thus, in idli batter the polymers produced by *L. mesenteroides*, *W. confusa*, *W. cibaria* and *Pediococcus parvulus*, positively affect the rheological properties, texture, mouth-feel, taste perception and the stability of fermented food (Kavitake et al., 2016).

Thus, it is clear that EPS produced by LAB already play a significant role in elaboration of cereal-based food and its contribution in the field will increase in the near future.

3. Aroma compounds produced by LAB

LAB help to improve the organoleptic properties of fermented food through synthesis of different compounds that cause changes in aroma, flavour and taste. Some LAB play a key role in cheese ripening converting components of milk to aroma and flavour compounds as a result of metabolism of carbohydrates, citrate, proteins and milk lipids.

Intact and lysed LAB contribute to the process of aroma formation during food fermentation. Cell lysis results in the release of cytoplasmic enzymes in the matrices of the fermentable food, and many of these retain their activity outside of the cell and will continue generating metabolites in the food matrix. It has been demonstrated that lysis of dairy starters-LAB plays an essential role in the process of cheese maturation (Lortal and Chapot-Chartier, 2005). Moreover, metabolism of fats by LAB enzymes is involved in the refining of cheeses affecting their organoleptic characteristics (Di Cagno et al., 2003). Also, catabolism of amino acids, present in the food matrices or generated by proteolysis, results in synthesis of aroma compounds. Branched-chain amino acids (Leu, Ile, Val) are converted into compounds contributing to malty, fruity and sweaty flavours; catabolism of aromatic amino acids, Phe, Tyr and Trp produce floral, chemical and faecal flavours; Asp is catabolized into buttery flavours and sulphur-containing amino acids (Met, Cys) are metabolized to compounds contributing to boiled cabbage, meaty and garlic flavours (Ardo, 2006). In addition, LAB produce aroma compounds by citrate metabolism and co-metabolism with sugars during various food fermentations. Therefore, in this section, we concentrate on the description of the features of the pathway to generate four carbon (C4) aroma compounds from citrate as well as flavour compounds generated by either proteolysis coupled to amino acid catabolism or to lipolysis.

3.1. C4 aroma compounds. A limited number of LAB can produce these compounds from citrate including *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (*L: diacetylactis*) (Hugenholtz, 1993), a few *Leuconostoc* (Hemme and Scheunemann, 2004), a few *Enterococcus* (Martino et al., 2016), *Lactobacillus plantarum* (Minervini et al., 2010), *Oenococcus oeni* (Bartowsky and Henschke, 2004) and *Weissella paramesenteroides* (García-Quintans et al., 2008).

Citric acid is an organic acid, which can be found in fermentable food products such as fruit, vegetables, fruit juices, milk and cheeses. Under anaerobic and acidic conditions, bacteria are able to metabolize citrate *via* pyruvate to produce the C4-carbon aroma compounds diacetyl, acetoin and 2,3-butanediol, as well as ethanol (Smid and Kleerebezem, 2014). These compounds can have a positive effect in dairy products such as butter and cottage cheese by providing typical nutty and buttery flavours (Martino et al., 2016).

L. diacetylactis and *Leuconostoc* species are the main citrate-fermenting LAB found in dairy starters (Drider et al., 2004), and their metabolism of this organic acid is a secondary proton motive force (PMF)-generating pathway (Lolkema et al., 1994). Citrate transport across the membrane is performed by the citrate permease P (CitP), which constitutes part of the 2-hydroxycarboxylate family of transporters for exchange of the dianionic form of citrate and lactate anions produced during sugar and citrate co-metabolism (Pudlik and Lolkema, 2012)(Fig. 1). Once inside the cell, citrate is cleaved into acetate and oxaloacetate by citrate lyase. Oxaloacetate is then converted to pyruvate and carbon dioxide by the action of the oxaloacetate decarboxylase. To relieve the stress of elevated levels of oxaloacetate, CitP eliminates the excess of this compound by exchange with citrate.

Insert Fig. 1

The C4 compound biosynthetic pathway starts by the condensation of two pyruvate molecules by acetolactate synthase to give α-acetolactate. This intermediate is readily decarboxylated to acetoin by α-acetolactate decarboxylase, or by non-enzymatic decarboxylation (in the presence of oxygen) to diacetyl. Acetoin can also be synthesised from diacetyl by diacetyl reductase. This enzyme has an acetoin reductase activity also (diacetyl/acetoin reductase), which forms 2,3 butanediol from acetoin (the reverse reaction being catalyzed by 2,3 butanediol dehydrogenase). Once synthesised, these C4

end-products are excreted from the cell without requiring specific transporters. Pyruvate can be also transformed to carbon dioxide and acetaldehyde thiamine pyrophosphate via thiamin pyrophosphate (TPP) (Laëtitia et al., 2014).

The efficiency of the diacetyl synthetic pathways depends on the activity of enzymes involved in the bioconversion of citrate to pyruvate, and the concentration gradient depends on the rate of disappearance of citrate in the intracellular medium (Laëtitia et al., 2014). Most of the pyruvate produced from citrate leads to the formation of lactate by lactate dehydrogenase (LDH) (Hemme and Scheunemann, 2004, Ko et al., 2016), and glucose/citrate co-metabolism in *L. diacetylactis* leads to the conversion of 80 % of the citrate metabolized to lactate (Smid and Kleerebezem, 2014). Thus, a strategy to increase production of diacetyl by re-routing or reinforcing the C4 compound metabolic pathway was one of the first metabolic engineering approaches applied to *L. lactis*. The most successful approach was the inactivation of α-acetolactate decarboxylase coupled with overexpression of NADH oxidase (Gaspar et al., 2013). However, the constructed recombinant strains have not been used for developing fermented products, mainly due to the concern of consumers regarding the use of genetically manipulated microorganism for food production.

The knowledge of citrate metabolism at the enzymatic and bioenergetic levels has been complemented with molecular biology studies. These revealed two types of transcriptional activation of transport of citrate and its conversion to pyruvate in LAB. In *L. mesenteroides* and *Weissella paramesenteroides* the expression of the plasmidic *citMCDEFGRP* operon encoding CitP and the enzymes for conversion of citrate to pyruvate are induced by the presence of citrate in the culture medium irrespective of the pH (Martin et al., 2005). This regulation, mediated by the CitI activator, together with the controlled cit operon, is present in other LAB (García-Quintans et al., 2008). In *L.*

diacetylactis, under acidic stress there is an induction of both the plasmidic *citQRP* operon (Garcia-Quintans et al., 1998), and of the chromosomal *citM-citI-citCDEFXG* cluster encoding citrate lyase and oxaloacetate decarboxylase (Martin et al., 2004). In addition, in *L. diacetylactis* the transcription of genes encoding the C4 compound biosynthetic pathway is also activated at low pH (García-Quintans et al., 2008).

All this knowledge in metabolism and genetics, together with functional genomics and its computational analysis, provide the basis for the detection of new strains and approaches to understand and control aroma formation from citrate in food fermentation processes.

3.2. Proteolysis and catabolism of amino acids. In addition to their ability to use sugars and citrate as source of carbon and energy, LAB can also use casein as a source of nitrogen. They have auxotrophies for various amino acids and they must be able to degrade proteins and peptides to satisfy their amino acid requirements.

Proteolysis is considered one of the most important biochemical processes involved in the manufacture of fermented dairy products and contributes to the development of their organoleptic properties (Chaves-López et al., 2011). The proteolytic systems of LAB are composed of: (i) proteinases, which hydrolyse caseins to give peptides, (ii) transport systems that translocate the peptides to the cytoplasm, and (iii) peptidases that convert peptides into amino acids. An example is the formation of flavour during cheese ripening in *L. lactis*, mainly due to proteolysis of the casein generating free amino acids, which act as substrates for catabolic reactions for generation of aroma compounds (Smid and Kleerebezem, 2014) (Fig. 2). In this bacterium, milk casein is hydrolysed by the cell envelope-associated serine proteinase PrtP to form oligopeptides which are transported into the cell cytoplasm by the Opp oligopeptides transport system.

Insert Fig. 2

Intracellular oligopeptides are then hydrolysed by a variety of peptidases to form amino acids.

Dipeptides and tripeptides and free amino acids, also present in milk, are transported by dipeptide, tripeptide transporters (DtpT, DtpP) and amino acid transporters, respectively. Dipeptides and tripeptides are further hydrolysed to amino acids. Serval studies have established that cheese made with strains deficient in proteinase activity lack flavour, have poor texture and age poorly. Cheese made with PepN or PepX (aminopeptidases) mutants was bitter and had low organoleptic quality (Hutkins, 2001).

The catabolism of amino acids into various alcohols, aldehydes, acids, esters and sulphur compounds, for specific production flavour profiles, depends on the activity of a variety of cytoplasmic enzymes (Smit et al., 2005)(Fig. 2). First, amino acids are converted to their corresponding α-ketoacid by the action of an aminotransferase. Recently, it has been shown that in *L. diacetylactis*, CitP is able to internalize α-ketoglutarate, the α-keto donor required for amino acid transamination to generate α-ketoacids, the first step in flavour development from excess amino acids in dairy fermentations (Pudlik and Lolkema, 2013). Thus, CitP seems to be a key player for two pathways of flavour compounds formation in *L. diacetylactis*.

Then, Ile, Leu, Val, Phe, Trp and Met are converted to their corresponding aroma compounds *via* the α-ketoacid pathways. The aldehydes generated by decarboxylation of α-ketoacids are substrate for either hydrogenases or dehydrogenases yielding respectively carboxylic acids and alcohols. Both can then be esterified by specific esterases, leading to the formation of thioesters or esters.

Methionine plays a major role in cheese flavour and its metabolism generates volatile sulphur compounds (methanethiol, dimethyldisulfide, dimethyltrisufide, 3-methylthio propanal, and thioesters) during cheese ripening. Studies of LAB strains have revealed

that in general lactococci have higher transaminase activities and superior yield of volatile sulphur compounds than lactobacilli and leuconostocs. Moreover, a great diversity in levels of aroma production has been detected intra- and inter-species, including detection of important aroma generation in probiotics LAB, such as *L. rhamnosus* GG and potentiation in mixed cultures with other microorganism (Martínez-Cuesta et al., 2013).

Finally, by decarboxylation among others amino acids, His, Tyr, Phe and Lys are converted respectively into histamine, tyramine, β-phenylethylamine, and cadaverin. These are biogenic amines, which do not contribute to a positive aroma, and if present at high concentration in the food can by hazardous for human health (Mohedano et al., 2015). Thus, it is very important to select and to combine strains for optimal aroma production without synthesis of the potentially harmful biogenic amines.

3.3. Lipolysis. The characteristic flavour of food products may also be due to the breakdown of lipids, Lipolysis like proteolysis, is directly related to flavour development and it is clear that the incorporation of lipolytic and/or proteolytic enzymes accelerate the flavour formation. Lipolysis generates free fatty acids and it is necessary to have a high concentration of these compounds to produce a perceptible effect on product flavour. Free fatty acids are produced from the de-esterification of triglycerides by esterase (Holland et al., 2005), and their subsequent metabolism generates many different compounds responsible for the aroma and texture such as esters, lactones, methyl ketones, alcohols and acids (Toldrá et al., 2001)(Fig. 2). The fatty acids can be converted by oxidation to: (i) hydroxyl-acids to produce lactone, and (ii) β-ketoacids which lead to methyl ketones and then to secondary alcohols. Also, hydroperoxide lyase cleaves free fatty acids to produce: (i) alcohols which are esterified

to produce esters, and (ii) acids. Finally, esterases can also catalyse the direct synthesis of esters from glycerides and alcohols via a transferase reaction in aqueous systems.

Most LAB possess only intracellular esterases, expect for *L. fermentum*, in which a surface-associated esterase has been isolated. As a result, most LAB esterases cannot hydrolyse food lipids until their release from lysed cells. This enzymatic activity varies according to the nature of lipids present and their degree of pre-hydrolysis. Esterases of LAB can also catalyse the direct synthesis of esters from glycerides and alcohols via a transferase reaction in aqueous systems (Thierry et al., 2016).

Lipolytic enzymes can produce different types of free fatty acids that are involved in cheesy, buttery and soapy flavour formation. The sensorial characteristics are strictly related to free fatty acid concentration, in serval products such as fermented milk. Different unpleasant flavours produced by LAB can be also attributed to lipolysis activity such as rancid, butyric, bitter, unclean, soapy and astringent (Barreto Penna et al., 2015).

Thus, it will be important to investigate fermentation process parameters as a tool for generation and modulation of aroma compounds formation from lipids, because excessive or unbalanced lipolysis caused by lipases or indigenous milk lipoprotein lipase could result in off-flavours. This may open new avenues to fine-tune flavour quality during fermentation for the delivery of new food product with attractive and distinguishable flavour to answer to consumer choice and preferences.

4. Polyols

Polyols form a sub-class of carbohydrates present in foods. They have a hydroxyl group (>CH-OH) instead of the carbonyl group (>C=O) present in some other carbohydrates. The name of the polyols usually uses the suffix ‘-itol’ instead of the suffix ‘-ose’ in agreement with modern carbohydrate nomenclature. ‘Sugar alcohol’ ‘sugar replacer’,

and ‘hydrogenated carbohydrate’ are terms that are often used to describe polyols. They are already used as sugar-free sweeteners and tooth-friendly food ingredients. Additionally it has been claimed that they are able to prevent or ameliorate diabetes and cardiovascular disease, the development of colon cancer, obesity and as well as acting as colon-hydrating, laxative and purifying agents (Gaspar et al., 2013). The best characterized polyols are: erythritol, xylitol, maltitol, isomaltitol, lactitol, polyglycitol, ribitol, arabitol, sorbitol and mannitol (Monedero et al., 2010, Livesey, 2003). In this section, we will discuss the role and metabolism of the two latter polyols.

4.1. Mannitol. This polyol, also designated mannite or manna sugar, is an important hexitol polyol used mainly in the food and pharmaceutical industries (Bhatt et al., 2013). It is widely distributed in nature, occurring in olive leaves, marine algae, fruits and vegetables (Oddo et al., 2002), in some bacteria (Saha and Racine, 2011) and in animals (Patra et al., 2011). The production of mannitol in animals is independent of insulin. It has a relative sweetness of 40–50 % compared to sucrose.

Toxicological studies have shown that mannitol does not have any significant adverse effect (Livesey, 2003, Kaialy et al., 2016) and, like other sugar alcohols, is included as a low-calorie ingredient in food products, which are currently used for the diet of diabetics (Ghoreishi and Shahrestani, 2009a, Ghoreishi and Shahrestani, 2009b). It has a low solubility in water of only 18 % (w/v) at 25 °C, particularly in alkaline solutions, and it is also used as a bodying and texturizing agent (Patra et al., 2011). Mannitol, as a common metabolite in microorganisms, contributes to the complex interactions between plants and pathogens (Guo et al., 2012), and provides to plants defense against fungal pathogens (Patel and Williamson, 2016), which may indirectly improve the quality of foods products made from plants.

Mannitol can be produced by chemical synthesis which involves hydrogenation and reduction reactions or by microbial synthesis or bioconversion using whole cells as the biocatalyst, a simple method that has been found most suitable for mannitol production due to its simplicity and robustness (Papagianni, 2012). Also, there is an alternative biocatalytic pathway, by which mannitol can be enzymatically produced with immobilized enzymes (Bhatt et al., 2013).

The chemical route for the synthesis of mannitol involves hydrogenation or other reductive reactions (Mazzoli et al., 2014). Mannitol can be produced in aqueous solution at high temperature (120 - 160ºC) with Raney nickel as a catalyst. In the catalytic hydrogenation reaction glucose and α-fructose units are reduced to sorbitol (the stereoisomer of mannitol) while β-fructose units are reduced to mannitol. The reduction reaction of fructose leads to mannitol and sorbitol in equal proportion. The reduction of sucrose under hydrolyzing conditions yields one part of mannitol and three parts of sorbitol, while, the reduction of glucose leads to mannitol (more than 20 % of glucose can be converted to mannitol) and the reduction of mannose with sodium borohydride or electrolysis leads to mannitol synthesis. Hydrogenation of a 50/50 fructose/glucose mixture produces an approximately 25/75 mixture of mannitol and sorbitol and the subsequent purification process to separate mannitol from its stereoisomer is difficult (von Weymarn et al., 2003, Ghoreishi and Shahrestani, 2009a, Ghoreishi and Shahrestani, 2009b).

Alternatively, mannitol can be produced by some microorganisms; this enzymatic process could have several advantages compared to the chemical synthesis. This property makes them suitable candidates for production of mannitol which form a common reserve or extracellular product of yeasts (*Candida mannitofaciens, Candida zeylanoides Candida magnoliae, Torulopsisversatilis*and*Torulopsisanomala*,

Rhodotorulaminuta), fungi (*Aspergillus* sp., *Penicilliumsp., Eurotiuem, Alternariaalternata, Cladosporiumherbarum, Epicoccumpurpurascens, Fusarium sp, Byssochlamysfulva*), red algea (*Caloglossaleprieurii*) and a few bacteria (*Pseudomonas putida, Escherichia coli, Bacillus megaterium, Gluconobacteroxydans*) (Monedero et al., 2010).

LAB can be also used for the production of low-calorie sugars (Patra et al., 2009). Among HeLAB, strains belonging to the *Leuconostoc, Oenococcus* and *Lactobacillus* genera are the most efficient microorganisms for mannitol production (Gaspar et al., 2013), they convert fructose directly into mannitol in a reaction catalyzed by mannitol 2-dehydrogenase (Hemme and Scheunemann, 2004), without producing sorbitol as by-product ((Otgonbayar et al., 2011) and Fig 3A). Theoretically in HeBAL, up to 2 mol of mannitol can be produced from 1 mol of glucose and 2 mol of fructose, when these sugars are co-metabolized (Saha and Racine, 2011). Depending on genus and species, different levels of mannitol production has been detected, and a *L. mesenteroides* strain was able to produce the polyol with a 67 % yield in a cashew apple juice supplemented medium containing only a final concentration of 50 g/L of reducing sugar (28 g/L of fructose) (Ortiz et al., 2013).

Insert Fig. 3

The expression of mannitol dehydrogenase appears to be constitutive in *Leuconostoc pseudomesenteroides*, whereas it is induced by growth on fructose in *Lactobacillus brevis* (Wisselink et al., 2002). *Leuconostoc sp* and *Lactobacillus sp.* isolated from kimchi have maximal mannitol production when fructose is the sole carbon source (Yun and Kim, 1998). *Lactobacillus florum* reached higher growth rates and final biomass in media containing both fructose and glucose, but the bacterium synthesised mannitol only when grown in the presence of fructose alone (Tyler et al., 2016).

HoLAB lack mannitol 2-dehydrogenase, consequently they are unable to produce naturally mannitol (Ortiz et al., 2013). However, they can utilize mannitol by the following pathway (Fig. 3B). They internalize mannitol by the action of a specific phosphoenolpyruvate phosphotransferase transport system (PTS-mannitol). Then, the intracellular mannitol 1-phosphate can be oxidized by mannitol 1-phosphate dehydrogenase to give the glycolysis-intermediate product fructose 6-phosphate.These catalytic reactions are reversible and in principle metabolic engineering could be applied to accomplish mannitol biosynthesis.

The glycolytic pathway is overall a reductive process and yields two molecules of NADH (Monedero et al., 2010). Bacteria need to maintain the redox balance by oxidizing NADH to NAD^+. For this, NADH is used to reduce pyruvate, producing lactate in HoLAB (Fig. 3B). Mannitol production could be considered as an alternative pathway to lactate synthesis for regeneration of NAD^+. Thus, genetic engineering has been applied to improve the efficiency of the mannitol biosynthetic pathway in HoLAB by generating LDH knock out mutants. Nuclear magnetic resonance analysis in resting cells revealed that a *L. lactis* LDH deficient mutant transiently accumulates high amounts of intracellular mannitol (up to 90 mM), but the polyol was further converted into mannitol 1-phosphate (up to 80 mM) (Neves et al., 2000). In addition, metabolic engineering was performed on the PTS-mannitol system of the *L. lactis* LDH deficient mutant. Disruption of *mtlA* and *mtlF*, which encode EIIA and EIIBC subunits, eliminated mannitol transport and resulted in a 33 % conversion of glucose to mannitol (Gaspar et al., 2004).

The current knowledge in the field indicates the future potential of LAB for mannitol production: (i) natural strains could produce high levels of mannitol *in situ* during food

manufacture by controlling fermentation conditions, and (ii) recombinant bacteria could be constructed for industrial production.

4.2. Sorbitol. This polyol, also named D-glucitol, is a six-carbon sugar alcohol which is naturally found in many fruits such as berries, cherries, plums, pears and apples. Due to its sweetness of about 60 % compare to sucrose and its high solubility in water, sorbitol is largely used in the food industry as a low-calorie sweetener, a humectant, texturizer or softener, being present in a wide range of food products such as chewing gums, candies, desserts, ice creams and diabetic foods. In addition, sorbitol is the starting material for the production of pharmaceutical compounds such as sorbose and ascorbic acid, and also is used as a vehicle for suspension of drugs (Silveira and Jonas, 2002). Furthermore, this polyol is poorly, or not absorbed in the small intestine, so it can reach the colon where it could be a potential substrate for bacterial fermentation, for this reason, sorbitol could be used as a prebiotic compound. In this sense, it has been demonstrated that the supplementation of the diet with sorbitol resulted in enrichment for lactobacilli in the rat colon and cecum (Sarmiento-Rubiano et al., 2007).

Traditionally sorbitol production has been by chemical catalytic hydrogenation of glucose or glucose/fructose mixtures (as detailed above for mannitol), but these processes often produce mixtures of sorbitol and mannitol which are difficult to separate, and thus increase the production cost (Mazzoli et al., 2014). Currently, only a few microorganisms, able to produce sorbitol naturally, have been described: the yeasts *Candida boidinii*, *Candida famata* and *Saccharomyces cerevisiae*, as well as the Gram-negative bacteria *Zymomonas mobilis*, but only the last offers the potential for industrial biotechnological production of sorbitol (Silveira and Jonas, 2002).

Currently, no LAB strain has been described to produce sorbitol naturally. But, there are some LAB (HeLAB and HoLAB), which have catabolic pathways for sorbitol

metabolism (Fig. 3). The enzymes involved in these pathways are encoded by genes organized in operons. Characterization of these operons in LAB was performed in *L. casei* (Alcantara et al., 2008) and *L. plantarum* (Ladero et al., 2007). Sorbitol is transported into the cells and phosphorylated to sorbitol-6P by the PTS-sorbitol system. Sorbitol-6P dehydrogenase catalyzes the conversion of sorbitol-6P to fructose-6P, which is introduced into the glycolytic pathway, but also this enzyme could catalyze the reverse reaction. Therefore, metabolic engineering could generate recombinant LAB strains able to synthesise sorbitol. Thus, a recombinant *L. casei* BL232 strain, with a conversion rate of glucose to sorbitol of 2.4 %,was obtained when the *gutF* gene, which encodes sorbitol-6P dehydrogenase, was integrated within the chromosomal *lac* operon (Nissen et al., 2005).

Sorbitol production by LAB, like mannitol production, would require high NADH levels for efficient reduction of sugar precursors, and as a consequence, when the *ldh l* gene, encoding LDH, of *L. casei* BL232 was inactivated the conversion rate of sorbitol synthesis by the bacteria increased to 4.3 % (Nissen et al., 2005). Also, in this bacterium it was demonstrated that when the *gutB* gene, that encodes the EIIBC component of the sorbitol PTS system, was deleted the reutilization of the produced sorbitol by the bacterium was avoided (De Boeck et al., 2010). This work demonstrated that sorbitol-6-P dephosphorylation and sorbitol excretion from the cells is carried out by enzymes as yet unknown. In addition, the nuclear magnetic resonance studies of the metabolism of this recombinant strain showed that production of mannitol in addition to sorbitol takes place. This problem was removed by inactivation of the *mtlD* gene that encodes the mannitol-1P dehydrogenase. This strain, under the best tested conditions, yielded a conversion rate of lactose into sorbitol of 9.4 % (De Boeck et al., 2010). Sorbitol

production efficiency in *L. casei* is far from the maximal theoretical yield of sorbitol from glucose, which is 67 %.

Two sorbitol operons, with two putative sorbitol-6-P dehydrogenase genes (*srlD1* and *srlD2*) were identified in the genome sequence of a *L. plantarum* NCIMB8826 strain (Ladero et al., 2007). Therefore, the srlD1/2 genes were cloned and overexpressed in a mutant strain deficient for both L- and D-lactate dehydrogenase activities. Both enzymes were demonstrated to be active and, using resting cells under pH control, achieved a conversion rate of 65 % of glucose to sorbitol, while in growing cells the conversion efficiency was of 25 %, perhaps due to a higher ATP demand for biomass production in the latter condition (Ladero et al., 2007). In this work, also a co-production of mannitol and sorbitol was obtained reaching up to 13 % conversion. That could be avoided by deleting native mannitol-1P dehydrogenase as in the case of *L. casei*.

The above results show the biotechnological potential of metabolic engineering of LAB strain for industrial sorbitol production, and usage as additive for the development of novel functional food.

5. Antimicrobial compounds

The use of LAB produced antimicrobial compounds as a barrier against pathogens, and food spoilage caused by bacteria has been proven to be effective. LAB produce a variety of compounds such as organic acids (lactic acid, acetic acid), diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins all of which have antibacterial or bacteriostatic properties. These LAB metabolic products not only extend shelf-life and inhibit the growth of pathogenic organisms but, as mentioned before, they have a positive effect on food taste, smell, colour, and texture (García et al., 2010, Zacharof and Lovitt, 2012). Nowadays, food producers face more challenges, because consumers

are more aware of the importance of food safety, demand higher quality and natural foods. The producers also have to heed government requirements to guarantee food safety. Also, there is concern about the use of manufacturing processes that involve thermal treatment (pasteurization, heating, sterilization), acidification, dehydration and addition of preservatives (antibiotics, nitrite, and sulphur dioxide; organic compounds such as propionate, sorbate, benzoate, lactate, and acetate). Although the aforementioned procedures are usually effective, there is an increasing public expectation for natural, microbiologically safe products providing the consumers with high health benefits (Zacharof and Lovitt, 2012). In this context, bacteriocins are becoming more attractive because they are obtained from natural sources and contribute to food safety (Yang et al., 2014). In this section, we will describe the current knowledge of bacteriocins synthesised by LAB and their role as bio-preservatives.

Bacteriocins are ribosomally synthesised antimicrobial peptides or proteins produced by a large variety of Gram-positive and Gram-negative bacteria, which inhibit or kill other microorganisms of closely related species to the producer and without affecting harmless microbiota (Cotter et al., 2013, Jack et al., 1995). Bacteriocins of Gram-positive bacteria are as abundant as and structurally more diverse than those produced by Gram-negative bacteria. Moreover, among Gram-positive, LAB are the most representative groups of bacteriocin-producing bacteria. Almost all LAB bacteriocins are non-toxic to eukaryotic cells and active in the nanomolar range (Messaoudi et al., 2013).

The bacteriocins kill bacteria at much lower concentrations than eukaryotic antimicrobial peptides, probably because they interact with a specific receptor present on target cells. For this reason, there is interest to use them in food. A property that supports the use of bacteriocins as food preservatives is that they are inactivated by

digestive proteases, and therefore have little influence on the gut microbiota. They are normally pH and heat-tolerant, they have no known cross resistance with other antibiotics, and they are usually encoded by genes located on a plasmid, facilitating genetic manipulation (Gálvez et al., 2007).

Bacteriocins can be bactericidal or bacteriostatic, their action depends on different factors such as bacteriocin dose and degree of purification, physiological state of the indicator cells and experimental conditions such as incubation temperature, pH or presence of agents disrupting cell wall integrity (Juodeikiene et al., 2012).

5.1. Bacteriocins from LAB. This is a very heterogeneous group and was first classified in the last decade of the 20th century (Klaenhammer, 1993), since then this has been a matter of debate (Heng and Tagg, 2006). Several authors have proposed different classifications based on different criteria as common characteristics, mainly primary structure, molecular weight, mode of action, heat stability and their genetic properties. According to Drider et al. (2006) (Drider et al., 2006), they can be divided into four classes:

Class I or lantibiotics, which are small thermostable peptides with a molecular weight lower than 5 kDa, and characterized by the presence of lanthionine and derivatives. Lantibiotics are subdivided in two groups: A, which includes elongated flexible molecules that act via membrane depolarization and B, which are globular in structure and interfere with cellular enzymatic reactions. The lantibiotics are represented by nisin and lacticin 3147 (Ross et al., 2002, Ryan et al., 1996).

Class II, which are small heat-stable peptides of <10 kDa. They are divided into three subclasses: IIa pediocin-like bacteriocins, including among others pediocin PA-1/AcH and some enterocins; IIb, bacteriocins composed of two peptides (lactocin G and plantaricin K) and IIc one peptide bacteriocins, with cyclic structures, whose prototype

is As48 enterocin. The classes III and IV include bacteriocins not well characterized. The class III is represented by high molecular weight (>30 kDa) thermosensitive peptides such as the helveticin J. Class IV is constituted by complex bacteriocins that require carbohydrate or lipid moieties for activity, they are circular heat-stable peptides with a head-to-tail peptide bond (Heng and Tagg, 2006, Franz et al., 2007).

The majority of bacteriocins produced by bacteria associated with food belong to class I or II and its number is growing continuously. The most representative with different and broad host target are shown in Table 2. Among them, nisin and pediocin PA-1/AcH are the most used as food preservatives. Nisin is undoubtedly the most well-known and characterized bacteriocin and the only one with widespread commercial application. It was approved for food applications by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 1969. It is the only bacteriocin approved for utilization as a preservative in many foods by the U.S. Food and Drug Administration (USFDA). Nisin is produced by some strains of *L. lactis* subsp. *lactis* and is composed of 34 amino acid residues (Gross and Morell, 1971).

Insert Table 2

Nisin can be effective at nanomolar concentrations depending on the target strain, possesses a broad spectrum of inhibition, and is active against numerous Gram-positive targets such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other pathogens and LAB species (Blay et al., 2007). It has also shown its efficacy against Gram-negative bacteria when it is utilised as an additional technological barrier (in the application of multiple processes or barriers) for food safety and preservation (Turgis et al., 2012). Finally, it has been broadly used in the food industry as an antibotulinic agent in cheese and liquid eggs, sauces and canned foods.

Nisin has a dual action mechanism: (i) to bind to lipid II hence impeding correct cell wall synthesis and (ii) to use lipid II, which is a membrane-bound precursor for cell wall

Insert Fig. 4

biosynthesis) as a docking molecule to start a process of membrane insertion and pore formation which leads to sudden cell death ((Balciunas et al., 2013) and Fig. 4).

Nisin synthesis is regulated by a two-component regulatory system comprising the membrane-bound histidine kinase sensor protein NisK and the regulator NisR. This regulatory system responds to extracellular nisin, which leads to the expression of genes involved in immunity and synthesis/posttranslational modification. This regulatory system is the basis for the nisin-induced controlled expression system (NICE) which is useful for the overexpression system of heterologous expression of many Gram-positive bacteria.

Pediocin PA-1 and nisin are the only bacteriocins commercially used as food preservatives. Pediocin PA-1 belongs to a group of bacteriocins produced by *Pediococcus* spp. It is a class IIA bacteriocin, where the mature structure has 44 amino acids, and contains the N-terminal consensus sequence YGNGVXC. Pediocin PA-1 is a highly hydrophobic, positively charged peptide, and is effective against many bacteria associated with food spoilage, such as *L. monocytogenes*. This is because it acts on the cytoplasmic membrane of target cells, recognizing receptors in the membrane-linked protein carrier of the PTS-mannose (Corsetti et al., 2015), and forms voltage-independent and hydrophilic pores through which important cellular metabolites are released, ultimately leading to cell death with or without lysis (Chikindas et al., 1993).

5.2. Applications of the BAL bacteriocins in the food industry. Foods, by definition are rich sources of nutrients and therefore susceptible to colonization by microorganisms, some of which may be harmful to human and animal health. To avoid the proliferation of pathogens, a possible solution is to incorporate bacteriocins in food. Their utilisation in food preservation may be advantageous in several aspects (Gálvez et al., 2007, Messaoudi et al., 2013): (i) to decrease the risks of food poisoning and transmission of

foodborne pathogens in the food chain; (ii) to extend the shelf life food products; (iii) to give extra protection during temperature abuse conditions; to reduce the use of chemical preservatives; (iv) to reduce wastage due to food spoilage; (v) to permit lower intensity physical treatments, thereby generating a better preservation of food nutrients and vitamins, and a better organoleptic properties of food, as well as decreasing of processing cost, and (vi) to supply alternative conservation barriers for "novel" foods and hopefully thereby satisfy the consumers demands (for safe, fresh-tasting, ready-to-eat, minimally-processed foods).

Bacteriocins can be incorporated into the food matrix through three different routes: (i) added directly to foods as purified or semi-purified antimicrobial additives (such as nisin), (ii) as bacteriocin-based ingredients from fermented foods (as for pediocin PA-1), and (iii) *in situ* production by inoculation of a producer strain in a fermenter. Also, it is possible to add immobilized bacteriocins in the food's packaging to extend the product life, or as food preservative (Cotter et al., 2005, Deegan et al., 2006, Gálvez et al., 2007, Mills et al., 2011).

A potential problem is that bacteriocins may not give enough protection against microbial contamination. Accordingly, several studies have revealed the potential efficacy of using bacteriocins in conjunction with other preservation methods (Mills et al., 2011). Therefore, the combination of bacteriocins with physical treatments such as high pressure processing or pulsed electric fields offer the possibility of more effective preservation of foods, also providing a further barrier to refractile types of contamination such as bacterial endospores (Gálvez et al., 2007). Also, it has been shown that a mix of nisin and high pressure homogenization allowed a decreased temperature treatment to prevent contamination of fruit juice by growth of *Listeria innocua*. (Pathanibul et al., 2009).

Additive or synergistic effects have been seen with several bacteriocins when used in combination with other antimicrobial agents. This can reduce microbial load in food products without diminishing its other qualities (Abriouel et al., 2002). The benefits of using mixtures of nisin and lysozyme (in proportion 1:2) against *L. monocytogenes* has been demonstrated (Chun and Hancock, 2000), now this blend is being used in biofilm for packaging, with EDTA as a third component: (Bhatia and Bharti, 2015). This mix is worthwhile from a financial point of view as lysozyme is 3-fold cheaper than nisin. Also, a combination of nisin with lacticin 3147 and polymyxin has been tested against *E. coli* and *E. faecium* (Draper et al., 2013). It was possible to decrease the concentration of added polymyxin in situations where the levels currently employed are of concern from a toxicity perspective. In addition, this mixture was able to inhibit some Gram-negative species. Furthermore, a synergism between bacteriocins and essential oils from *Origanum vulgaris*, *Thymus vulgaris* and *Satureja montagna* has been observed against food pathogenic bacteria such as *E. coli* O157:H7, *L. monocytogenes* and *S. typhimurium* (Turgis et al., 2012).

In conclusion, the bacteriocins are attractive candidates for use as biopreservatives in the food industry due to their spectrum of inhibition, bactericidal mode of action, and relative tolerance to technologically relevant conditions (pH, NaCl, heat treatments). The application of bacteriocins and peptides in foods has the potential to prolong shelf-life, and increase food safety, inhibiting pathogenic and spoilage bacteria, avoiding or reducing the use of undesirable preservatives in food. However, it should be pointed out that the use of bacteriocins as ingredients or additives requires new strategies for large scale production in suitable low-cost food-grade media.

6. Vitamin production by LAB

Vitamins are micronutrients essential for many biological processes, which take place in

all living organisms. They are often classified into two groups: (i) fat-soluble, which includes vitamins A, D, E and K; and (ii) water-soluble, which comprise vitamin C and the B-group vitamins including thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7 or H), folate (B11–B9 or M) and cobalamin (B12). Most of these vitamins are not synthesised by humans, and they must be acquired exogenously from the diet. In addition, in the gut microbiota certain strains of LAB can synthesise vitamins allowing their uptake from the digestive tract (LeBlanc et al., 2013). Some vitamins are easily removed or destroyed during cooking and food processing, so insufficient intake is common in many societies. Thus, deficiency of these vitamins is a problem nowadays in many countries including highly industrialized nations mainly due to unbalanced diets (LeBlanc et al., 2011). For this reason, several countries have adopted laws requiring the fortification of certain foods with specific vitamins and minerals (LeBlanc et al., 2011). For this purpose, the classical method was to supplement food with vitamins chemically synthesised. However, microorganisms are also an important source of vitamins, since they are able to produce them especially B-group vitamins such as riboflavin, folate and cobalamin (Burgess et al., 2009, LeBlanc et al., 2011). Therefore, in the last decades chemical vitamin synthesis has been replaced by fermentation production, and for some vitamins like riboflavin this method currently represents the whole world production (Schwechheimer et al., 2016).

In addition, the current challenge is to enhance the *in situ* fortification of fermented foods by vitamin-producing LAB that could result in a more natural and therefore consumer-acceptable alternative (LeBlanc et al., 2011, Capozzi et al., 2012). Cereals may contain variable amount of B-vitamins, but their viability can be decrease due to industrial processing or other factors. Cereals are optimal substrates for fermentation by LAB (Capozzi et al., 2012), and this offers opportunities to improve the nutritional

value of food products, compensating for any losses that may have occurred during the manufacturing processes. Thereby, it permits the development of novel functional foods with enhanced vitamin content due to LAB fermentation (Capozzi et al., 2011, Russo et al., 2014, Russo et al., 2016).

In this review we focus on the current knowledge on LAB producing the B-group vitamins, riboflavin and folate, and their biotechnological applications.

6.1. Riboflavin. Vitamin B2 is involved in many essential metabolic cellular processes. It is the precursor of the flavocoenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), cofactors for flavoproteins acting as electron carriers in a wide variety of redox reactions (Fischer and Bacher, 2005). The biosynthesis of one riboflavin molecule requires one molecule of guanosine triphosphate (GTP) and two molecules of ribulose 5-phosphate as substrates. The biosynthesis of riboflavin consists in seven enzymatic steps and it has been extensively reviewed elsewhere (Fischer and Bacher, 2005, Fischer and Bacher, 2008, Haase et al., 2014).

Riboflavin is present in foods of animal origin like liver, egg yolk, milk and meat, and also in vegetable and cereal-based foods (Capozzi et al., 2011). It is essential for humans, and the recommended daily intake of riboflavin is 1.3 mg per day for men and 1.1 mg per day for women. Despite the wide range of food containing riboflavin, its availability is low and its deficiency (ariboflavinosis) persists in both developing and industrialized countries. Riboflavin status in humans is determined by the erythrocyte glutathione reductase activation coefficient (EGRAC), which is the ratio between glutathione reductase activity determined with and without the addition of the cofactor, FAD and values higher than 1.3 indicate a suboptimal riboflavin status, and it has been reported that in the UK population, 82 % of men and 77 % of women aged 19 to 24 years old present EGRAC values > 1.3 (McNulty and Scott, 2008).

Riboflavin deficiency can lead to liver and skin damage, and changes in cerebral glucose metabolism, with symptoms like hyperemia, sore throat, edema of oral and mucous membranes, cheilosis and glossitis (LeBlanc et al., 2011).

For this reason, many countries have programs for fortification of riboflavin by improved feed and food. Fermentative microorganisms producing riboflavin have replaced chemical synthesis, and the most important industrial producers are *Bacillus subtilis* and *Ashbya gossypii* (Schwechheimer et al., 2016). In bacteria the biosynthetic pathway is encoded by the *rib* operon, and regulation of its expression has been extensively studied in the Gram-positive bacterium *B. subtilis* (Schwechheimer et al., 2016). In addition, for productive exploitation, metabolic engineering of this bacterium has been performed (Schwechheimer et al., 2016). Exposure to roseoflavin, a riboflavin analogue yielded overproducing mutants, which had a deregulated *rib* operon due to mutations in *ribC*, a *trans*-acting regulator of the operon. Furthermore, strategies for *rib* operon duplication as well as substitution of its promoter, even the overexpression of *ribA* have been driven to achieve more commercially competitive *B. subtilis* strains. As a consequence, currently riboflavin produced by metabolically engineered *B. subtilis* can be used as a food and feed additive and also be used by the pharmaceutical industry. Recently, several studies have indicated the potential use of riboflavin for the treatment of diseases such as headache (Schetzek et al., 2013), migraine (Sherwood and Goldman, 2014), anemia (Shi et al., 2014) and neurodegenerative disorders (Foley et al., 2014).

LAB can synthesise or utilize riboflavin during food fermentation, so an adequate selection of strains is needed to increase the concentration and bioavailability of this essential vitamin in fermented foods. To this end, the use of roseoflavin for selection of spontaneous roseoflavin-resistant mutants was found to be a reliable method to select natural riboflavin-overproducing strains of various species (*L. plantarum*, *L. lactis*, *L.*

mesenteroides and *Propionibacterium freudenreichii*) commonly used in the food industry (Burgess et al., 2004, Burgess et al., 2006). Two fermented dairy products made with the natural vitamin B2-overproducing *L. lactis* strain (LeBlanc et al., 2005) and *P. freudenreichii* strain (LeBlanc et al., 2006) have been reported to counteract riboflavin deficiency in an animal model.

Moreover, cereal-based products have been suggested as matrices for a number of new formulations of vitamin enriched functional food by LAB fermentation to increase health benefits (Capozzi et al., 2012) and in fact, previously, LAB strains were isolated from durum wheat flour and were treated with roseoflavin to achieve natural riboflavin-overproducing strains. Two *L. plantarum* roseoflavin-resistant strains were used for fermenting sourdough for the preparation of bread and pasta, resulting in an appreciable increase of riboflavin content (about 2- and 3-fold increases in pasta and bread, respectively) (Capozzi et al., 2011). Furthermore, a *Lactobacillus fermentum* strain isolated from sourdough was found to produce riboflavin and was used to fortify bread increasing by 2-fold the riboflavin content of bread (Russo et al., 2014).

The use of oat-based food is an alternative for the development of new functional food. Oat beverages fermented with the riboflavin-overproducing *L. plantarum* LpB2 strain showed a 2-fold increase in riboflavin content, and this concentration further increased during cold storage (Russo et al., 2016). In addition, the LpB2 strain showed probiotic properties such as gastro-intestinal stress tolerance and *in vitro* adhesion to human Caco-2 cells and inhibition of pathogens (Arena et al., 2014). Also, the LpB2 strain has the ability to colonize *in vivo* the gut of gnotobiotic zebrafish larvae (Russo et al., 2015). Colonization was visualized *in vivo* by fluorescently labelling the strain with mCherry protein encoded by the *mrpf* gene carrying the pRCR12 plasmid and based on the expression vector pRCR (Mohedano et al., 2015).

6.2. Folate. Vitamin B11 is the term used to describe all folic acid derivatives including the polylglutamates naturally present in foods, and folic acid that is a synthetic folate form, which is commonly used for food fortification and nutritional supplements. Folate is an essential vitamin. It functions as a cofactor for the transfer of one-carbon units from donor molecules to biosynthetic pathways leading to methionine, purine, and pyrimidine biosynthesis. Thus, folate is involved in many metabolic pathways such as the biosynthesis of DNA and RNA and the inter-conversions of amino acids. Moreover, folate possesses antioxidant capacity that protects the genome by preventing free radical damage of DNA (Burgess et al., 2009, Iyer and Tomar, 2009, LeBlanc et al., 2011).

Dietary folate can be found in legumes, leafy greens, some fruits and vegetables as well as in liver and fermented dairy products such as yogurt, where its concentration could be increased depending on the starter cultures used and the storage condition. Deficiency of folate in humans may contribute to several health disorders including Alzheinmer's disease, coronary heart diseases, osteoporosis, growth retardation, megaloblastic anemia, congenital malformations, increased risk of breast and colorectal cancer and neural tube defects in new born infants (Iyer and Tomar, 2009, LeBlanc et al., 2011).

The recommended daily intake of folic acid is 400 μg of dietary folate equivalents. This value is defined based on the differences between the bioavailability of natural folates and synthetic folic acid added to the food, which is assumed to be less than 50% of the bioavailability of natural folates relative to folic acid (McNulty and Scott, 2008). This is due to the fact that natural food folates (polylglutamates) must be hydrolyzed to monoglutamate forms prior to absorption in the small intestine (Iyer and Tomar, 2009). Furthermore, folic acid provides a more stable form of the vitamin (McNulty and Scott,

2008). However, high level intake of chemically produced folate has been shown to cause severe health defects such as vitamin B12 deficiency, which is a risk for pregnant woman, and induces cancers. Thus, the rationale would suggest focusing on naturally produced folate for food fortification (Iyer and Tomar, 2009).

Several LAB are able to synthesise folate. The biosynthetic route has seven steps, which convert guanosine triphosphate to tetrahydrofolate. *De novo* synthesis of folate requires the precursors 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) and para-aminobenzoic acid (pABA) (Rossi et al., 2011). Metabolic engineering has increased folate production in *L. lactis* (Wegkamp et al., 2007). Also, the use of the folate antagonist methotrexate in strains of *L. plantarum*, *L. fermentum* and *E. faecium*, previously identified from durum wheat semolina, identified potential folate-overproducing strains usable for cereal fermentation (Capozzi et al., 2012).

Fermented dairy products are suitable targets for folate fortification. The presence of folate-binding proteins in milk may contribute to increase efficiency of folate human absorption by protecting dietary folates from uptake by bacteria in the gut. Several attempts were carried out to exploit strains of *Lactobacillus* for this aim, but unfortunately, most *Lactobacilli* species are unable to produce folate in the absent of pABA, with the exception of *L. plantarum* (LeBlanc et al., 2013, Rossi et al., 2011). On the contrary, most of these bacteria, like *L. bulgaricus,* are folate consumers reducing folate level in food. So, it is important to select adequate starter cultures for increasing folate concentrations. A yogurt starter *S. thermophilus* strain was reported to produce folate. The use of this strain in combination with other producer strains like *L. lactis* or *Leuconostoc* species significantly increased folate levels (LeBlanc et al., 2011). Folate-overproducing strains of *L. bulgaricus* and *S. thermophilus* species from artisanal Argentinean yogurts have been isolated and this was the first report where natural

strains of *L. bulgaricus* were shown to produce folate (Laiño et al., 2012). Using some of these strains as starter cultures a yogurt was produced with a high folate concentration (180 μg/L), which implies almost a 250 % increase with respect to the non-fermented milk (Laiño et al., 2013). Recently, these authors, by supplementation of the starters with a *Lactobacillus amylovorus* strain, increased the folate concentration in the yogurt to 263 μg/L (Laiño et al., 2014). This was the first report where a *L. amylovorus* strain was successfully used as co-culture for natural folate bio-enrichment of fermented milk. In other work, a *L. lactis* folate-producing strain was used to fortify skimmed milk, increasing the folate concentration to 129 μg/L (Divya and Nampoothiri, 2015). Also, LAB folate-producing strains have the potential to fortify non-dairy fermented food. *S. thermophilus* and *L. reuteri* among other folate-producing microorganisms have produced folate enriched oat and barley fermented food (Kariluoto et al., 2014).

Finally, several bifidobacteria species have been verified to produce folate and were used to produce fermented food (D'Aimmo et al., 2012). The administration of bifidobacteria high-producing folate strains was shown to cause an increased faecal level of folate in both rats and humans (LeBlanc et al., 2013).

Thus, the production of vitamins by LAB in dairy- and cereal-based products seems to have a promising potential for the food industry and it is expected that it will develop very fast in the coming years. The challenge in the field is to get commercially competitive products into the market.

7. Conclusions and perspectives

It is obvious that many of the LAB metabolic pathways (central and secondary) are continuing to be important for the preparation of traditional fermented products, with

the LAB metabolites contributing to the quality and safety of food and beverages. In addition, metabolic engineering of LAB has detected and developed strains overproducing metabolites such as EPS, vitamins and bacteriocins that are currently used to generate food additives. Presumably, these lines of research will continue to be developed: for example commercially competitive production of polyols by LAB is expected to be achieved. With regard to the organoleptic properties of the fermented food, a combination of different natural strains is the best option to reach an optimal metabolic network of different pathways. For this, the current omics techniques as well as bioinformatics analysis of the genome of the starters and co-adjuvant bacteria will help to establish an optimal consortium for getting the expected texture and flavour in various food matrices. Finally, the most promising future contribution of the LAB metabolites for the food industry will be in the field of development of functional food by *in situ* production (e.g. polysaccharides and vitamins), not only in the dairy sector but also in the sectors such as cereal- and meat-based products. Currently there is not a good consumer-acceptance of the use of genetically manipulated microorganism in food preparation, so it will be necessary to detect and validate the use of naturally overproducing bacteria or select spontaneous mutants to increase the development of commercially competitive enriched food (e.g. with vitamins) by *in situ* fermentation.

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Legend to the figures

Figure 1. C4 aroma compounds biosynthesis in LAB. ALD, acetolactate decarboxylase; ALS, α-acetolactate synthase; BDH, 2,3 butanediol dehydrogenase; CL, citrate lyase; CitP, citrate permease P; CoA, coenzyme A; DAR, diacetyl/acetoin reductase; DS, diacetyl synthase; LDH, lactate dehydrogenase; OAD, oxaloacetate decarboxylase; TPP, thiamine pyrophosphate; Dashed lines, non-enzymatic reaction.

Fig. 2 . Biosynthesis of flavour compounds by proteolysis coupled to amino acid catabolism and lipolysis in LAB. AA, amino acids; AAT, amino acid transporters; CitP, citrate permease P; Opp: oligopeptides transport system; PrtP, cell envelope-associated proteinase.

Fig. 3. Pathways for mannitol, sorbitol, glucose and fructose metabolism of HeLAB (A) and HoLAB (B). PTS, phosphoenolpyruvate phosphotransferase systems for fructose , glucose, mannitol and sorbitol; AK, acetate kinase; GPI, glucose phosphate isomerase; PGI, phosphoglucose isomerase; LDH, lactate dehydrogenase; M2D, mannitol-2 dehydrogenase M1PD, mannitol-1- phosphate dehydrogenase; M1P, mannitol-1 phosphatase; S6PD, sorbitol-6-phosphate dehydrogenase; XPK, xylulose-5P phosphoketolase; ?, unknown enzyme/s, *, several enzymatic reactions.

Figure 4. Mode of action of nisin. The N-terminus of nisin A binds the pyrophosphate of lipid II and permeabilizes the plasma membrane, resulting in pore formation, which allows the efflux of intracellular molecules).

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Table 1. Exopolysaccharides from LAB in food.

FOOD	ORGANISMS	EPS	REFERENCES
Yogurt	*Lactobacillus delbrueckii subsp. bulgaricus* *Lactobacillus mocosae* *Streptococcus thermophilus*	HePs	(London et al., 2015, Folkenberg et al., 2006)
	Lactobacillus johnsonii *Lactobacillus reuteri* *Leuconostoc citreum* *Streptococcus mutans*	Inulin	(Balthazar et al., 2016)
Kefir	*Lactobacillus kefiranofaciens* *Lactococcus lactis ssp.cremoris* *Lactococcus lactis ssp.lactis* *Leuconostoc mesenteroides* *Streptococcus thermophilus*	Kefiran	(Maeda et al., 2004, Hamet et al., 2015)
Cheese	*Lactobacillus casei* *Lactobacillus delbrueckii subsp. bulgaricus* *Lactobacillus helveticus*	HePs	(Broadbent et al., 2001)
	Lactobacillus johnsonii *Lactobacillus reuteri* *Leuconostoc citreum* *Streptococcus mutans*	Inulin	(Karimi et al., 2015)
Viili	*Lactococcus lactis ssp. cremoris* *Lactococcus lactis ssp. lactis*	HePs	(Kahala et al., 2008)
Bakery products	*Lactobacillus frumenti* *Lactobacillus panis* *Lactobacillus pontis* *Lactobacillus reuteri* *Lactobacillus sanfranciscensis* *Leuconostoc mesenteroides* *Streptococcus mutans* *Streptococcus salivarius*	Levan	(Bounaix et al., 2009, Galle et al., 2010, Katina et al., 2009)
	Lactobacillus reuteri	Reuteran	(Bounaix et al., 2009)
	Leuconostoc citreum *Leuconostoc mesenteroides* *Streptococcus mutans* *Weisella cibaria* *Weisella confuse*	Dextran	(Kajala et al., 2016, Kajala et al., 2015, Kavitake et al., 2016)
	Lactobacillus buchneri	HePs	(Galle et al., 2011)

Table 2. Broad host spectrum bacteriocins from LAB and their target.

BACTERIOCINS	PRODUCER	TARGET	TARGET ORGANISMS*	REFERENCES
Nisin A	*Lactococcus lactis subsp. lactis*	Lipid II/pore formation	*Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Listeria, Clostridium*	(Gross and Morell, 1971)
Lacticin 3147	*Lactococcus lactis*	Depolarization cellular membrane/Lipid II	*Enterococcus, Lactobacillus, Lactococcus, Leuconostoc*	(Ryan et al., 1996)
Pediocin PA-1/ACH	*Pediococcus Acidilactici* PAC1.0	Man-PTS/Pore forming	*Pediococci, Lactobacilli, Leuconostoc, Brochothrix thermosphacta, Propionibacteria, Bacilli, Enterococci, Staphylococci, Listeria clostridia, Listeria monocytogenes, Listeria innocua*	(Chikindas et al., 1993)
Plantaricin K	*Lactobacillus plantarum*	Lipid II	*Pediococcuspentosaceus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus sakei, Lactobacillus viridescens, Pediococcuspentosaceus, Carnobacteriumpiscicola, Lactobacillus plantarum, Lactobacillus sakei, Pediococcusacidilactici, Lactobacillus curvatus*	(Gálvez et al., 2007)
Enterocin AS-48	*Enterococcus faecalis* AS-48	Non-specific pore forming	*Bacillus subtilus, Bacillus cereus, Bacillus circulans, Bacillus megaterium, Corynebacterium glutamicum, Corynebacterium bovis, Mycobacterium phlei, Nocardiacorrallina, Micrococcus luteus, Micrococcus lysodeikticus, Staphylococcus aureus, Streptococcus faecalis, Streptococcus faecium. Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus inconstans, Salmonella typhimurium, Shigellasonnei, Pseudomonas fluorescens , Pseudomonas aeruginosa, Pseudomonas reptilivora*	(Abriouel et al., 2002)

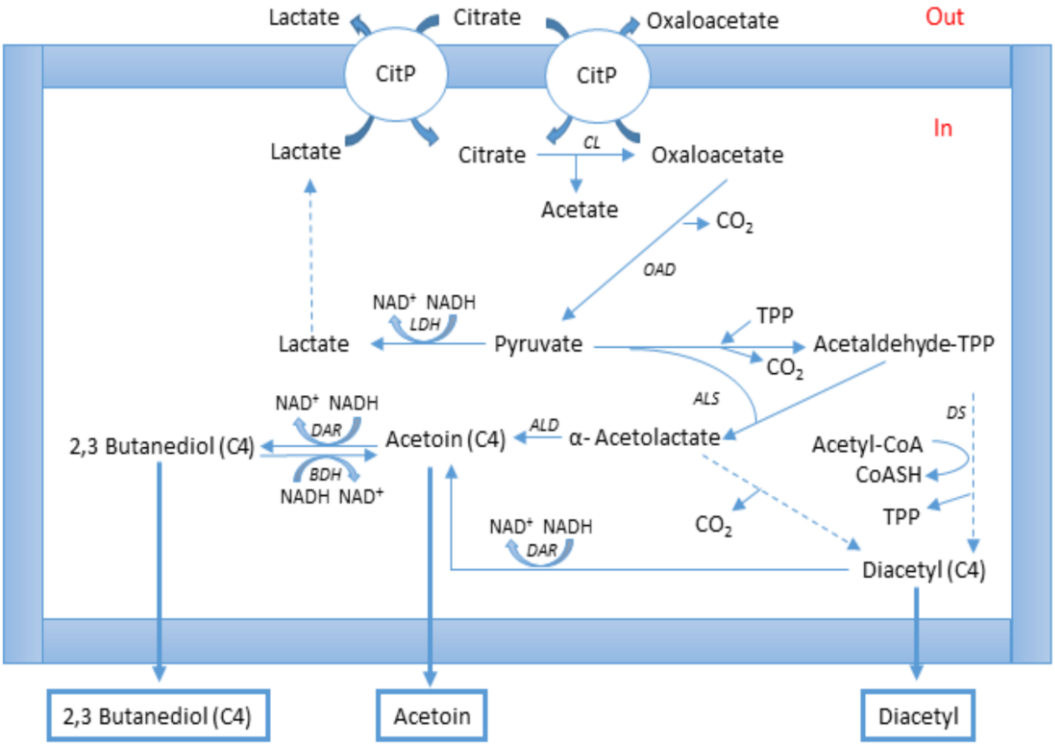
Lactate
Citrate
Oxaloacetate
Out
CitP
CitP
In
Lactate
Citrate
CL
Oxaloacetate
Acetate
CO_2
OAD
NAD⁺ NADH
LDH
TPP
Lactate
Pyruvate
Acetaldehyde-TPP
CO_2
ALS
DS
NAD⁺ NADH
DAR
2,3 Butanediol (C4)
Acetoin (C4)
ALD
α- Acetolactate
Acetyl-CoA
CoASH
BDH
NADH NAD⁺
TPP
CO_2
NAD⁺ NADH
DAR
Diacetyl (C4)
2,3 Butanediol (C4)
Acetoin
Diacetyl

Figure 1

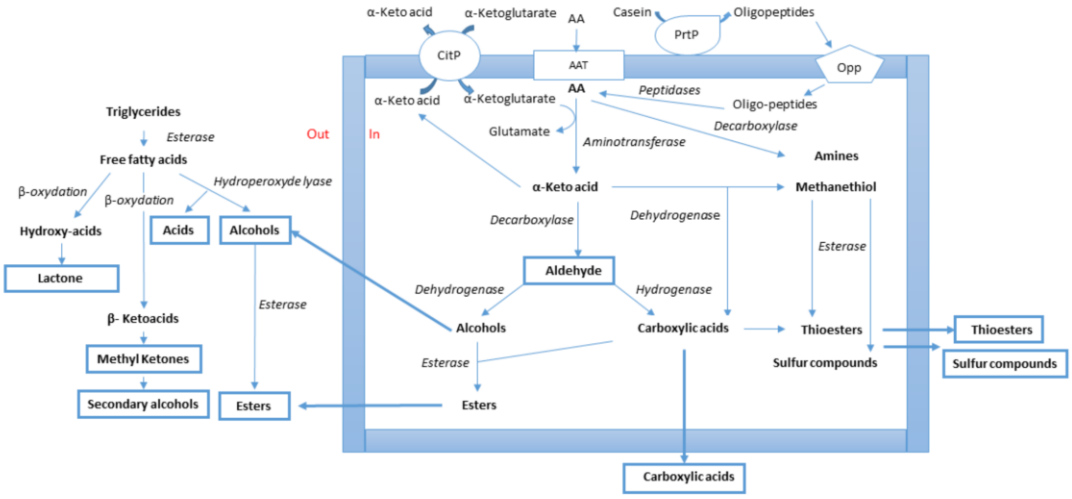
α-Keto acid
α-Ketoglutarate
AA
Casein
Oligopeptides
PrtP
CitP
AAT
Opp
AA
Peptidases
Oligo-peptides
α-Keto acid
α-Ketoglutarate
Glutamate
Decarboxylase
Aminotransferase
Amines
Triglycerides
Esterase
Out
In
Free fatty acids
α-Keto acid
Methanethiol
β-oxydation
β-oxydation
Hydroperoxyde lyase
Decarboxylase
Dehydrogenase
Hydroxy-acids
Acids
Alcohols
Esterase
Lactone
Aldehyde
Dehydrogenase
Hydrogenase
Esterase
β- Ketoacids
Alcohols
Carboxylic acids
Thioesters
Thioesters
Esterase
Sulfur compounds
Sulfur compounds
Methyl Ketones
Secondary alcohols
Esters
Esters
Carboxylic acids

Figure 2

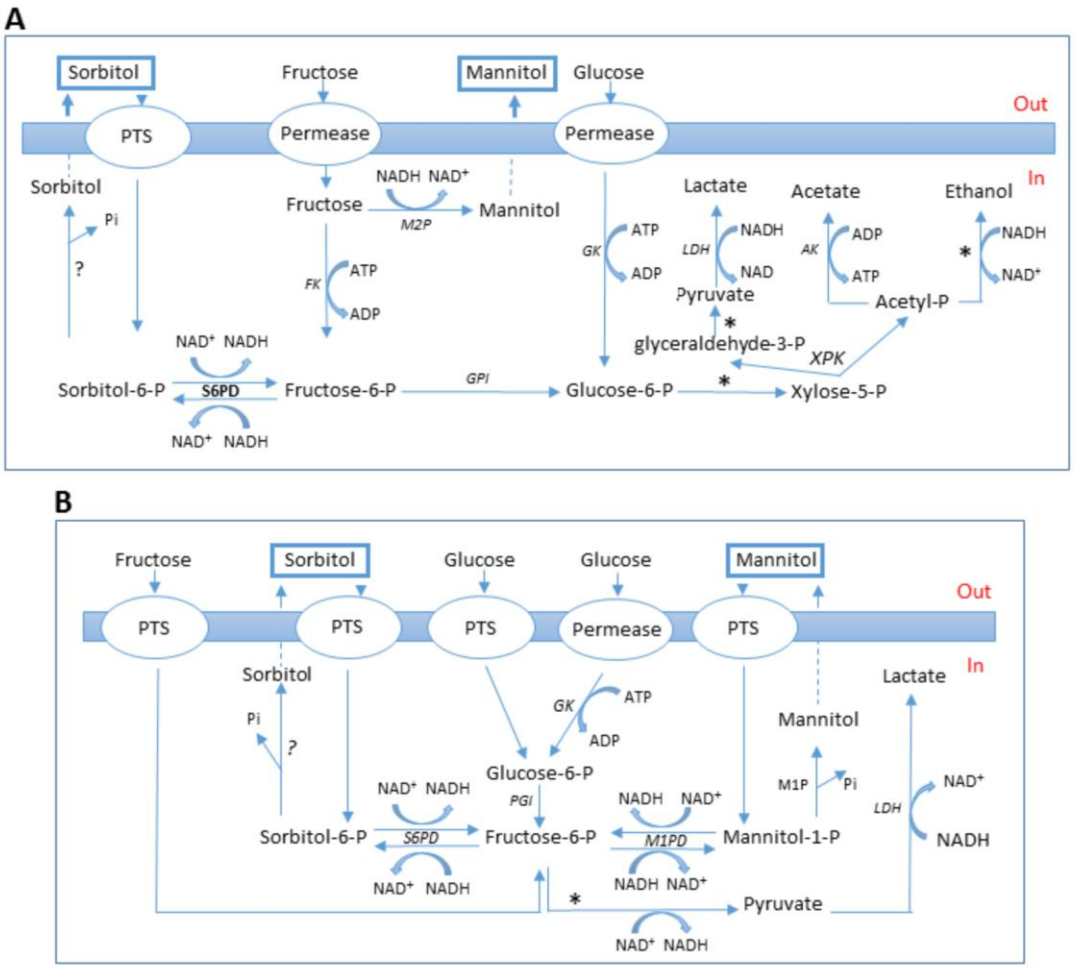
A
Sorbitol
Fructose
Mannitol
Glucose
Out
PTS
Permease
Permease
In
Sorbitol
Pi
?
Fructose
NADH NAD+
M2P
Mannitol
FK
ATP
ADP
GK
ATP
ADP
Lactate
LDH
NADH
NAD
Pyruvate
Acetate
AK
ADP
ATP
Acetyl-P
Ethanol
NADH
NAD+
glyceraldehyde-3-P
XPK
NAD+ NADH
Sorbitol-6-P
S6PD
Fructose-6-P
GPI
Glucose-6-P
Xylose-5-P
NAD+ NADH
B
Fructose
Sorbitol
Glucose
Glucose
Mannitol
Out
PTS
PTS
PTS
Permease
PTS
In
Sorbitol
Pi
?
GK
ATP
ADP
Glucose-6-P
Mannitol
Lactate
NAD+ NADH
PGI
NADH NAD+
M1P
Pi
LDH
NAD+
NADH
Sorbitol-6-P
S6PD
Fructose-6-P
M1PD
Mannitol-1-P
NAD+ NADH
NADH NAD+
Pyruvate
NAD+ NADH

Figure 3

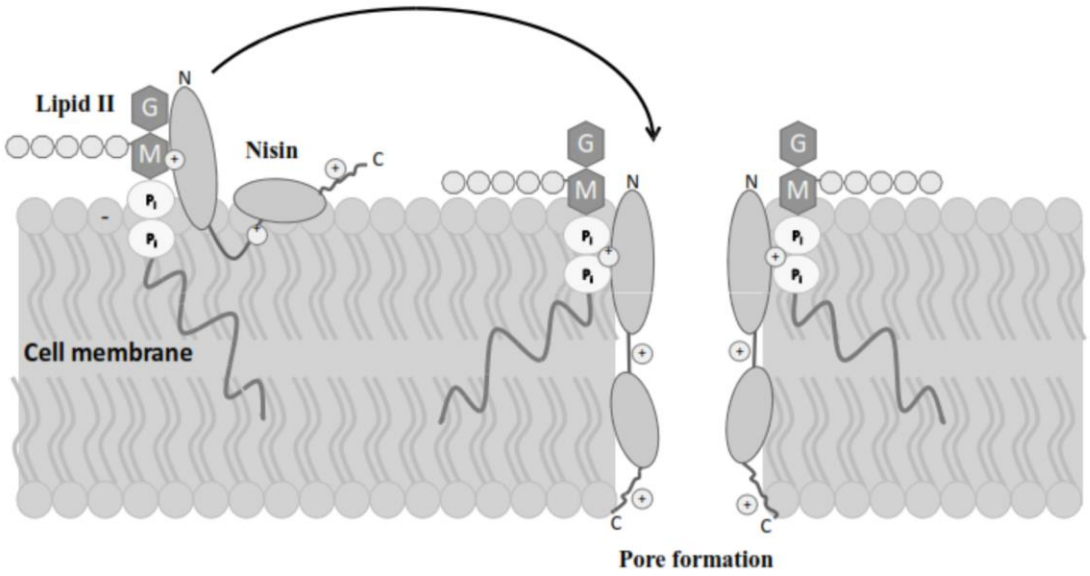

Figure 4