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REVIEW PAPER

Bacterial L-leucine catabolism as a source of secondary metabolites

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Abstract L-Leucine can be assimilated by bacteria when sugars or other preferential carbon sources in the habitat are depleted. The L-leucine catabolism is widely spread among bacteria and has been thoroughly studied. Its pathway is comprised by multiple reactions and converges with other catabolic routes, generating acetoacetate and acetyl-CoA as its final products. The initial three steps are conserved in most bacteria, constituting the first steps of the branched-chain amino acids catabolic pathway. The main product of these sequential reactions is the 3-methylcrotonyl-CoA metabolite, which undergoes further enzymatic steps towards the production of acetoacetate and acetyl-CoA. These, however, are not always the final products of L-leucine catabolism, as intermediates of the pathway can further synthesize fatty acids or feed other secondary metabolism pathways in order to produce diverse compounds which can exhibit biological activities. This alternative metabolism typically leads to the accumulation of products bearing industrial relevance, including volatile compounds used in the food industry, compounds with antimicrobial activity, production of biofuels and biopolymers. In anaerobic bacteria, the L-leucine catabolism may induce the accumulation of a variety of organic compounds acids, such as isovaleric, isocaproic, and 2-methylbutyric acids. In conclusion, the usage by bacterial species of L-leucine as an alternative carbon and nitrogen source may contribute to their environment adaptability and, more importantly, the diverse products that can be obtained from L-leucine metabolism may be represent a valuable source of compounds of biotechnological interest.

Keywords L-Leucine metabolism · α-Ketoisocaproic acid · Isovaleric acid · Secondary metabolites · Branched-chain amino acids

1 Introduction

During the appearance of life in our planet, L-leucine arose on Earth or came from space, becoming one of the twenty amino acid that form proteins in all living organisms (Lazcano and Miller 1999; Brack 2007). At present, L-leucine has an overall prevalence of 9.1 %, positioned as the most abundant amino acid in proteins. As such, it appears to be a suitable reporter molecule for monitoring the availability of substrates for protein and peptide synthesis, as well as surveying

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amino acids in ecosystems (Brinkman et al. 2003; Dai et al. 2012; Scully et al. 2015). This capability is conferred by the Leucine-responsive regulatory protein (Lrp), which modulatesthat is capable to recognize L-leucine by leading to modulation of transport and metabolism of amino acids; thus, L-leucine has been used as a reporter molecule for the monitoring of bacterial growth in aquatic ecosystems (Kirchman et al. 1985; Brinkman et al. 2003; Del Giorgio et al. 2011; Scully et al. 2015). L-Leucine catabolism is widely spread among bacteria and generates intermediary products that feed routes of central metabolism, therefore representing a carbon and nitrogen source that supports bacterial growth (Ganesan et al. 2006; Kazakov et al. 2009).

L-leucine is mainly assimilated through the L-leucine catabolic pathway (Fig. 1), which has a significant biological and biotechnological importance, marked by the biodegradation of recalcitrant compounds (Lahme et al. 2012; Schuster et al. 2012; Hyman 2013) and the generation of intermediates for synthesizing metabolites with wide-range applications in medical, food, bioplastic and biofuel industries (Fig. 2) (Yan and Liao 2009; Ziadi et al. 2010; Saika et al. 2014; Vogt et al. 2015). The intermediates α-ketoisocaproic acid and isovalery-CoA, generated during the L-leucine catabolism, are of special interest as they are precursors of a wide range of compounds; they can be channeled to alternate routes of secondary metabolism to produce compounds with biotechnological applications (Zhang et al. 2008; Smit et al. 2009). The α-ketoisocaproic acid is a regulation factor in metabolism which has been used in the therapy for treating chronic kidney disease patients (Zhu et al. 2011; Vogt et al. 2015), whereas in food and fuel industries it is a precursor of flavor compounds and biofuels, respectively (Ziadi et al. 2010; Sinz and Schwab 2012). On the other hand, isovalery-CoA plays an important role in living organisms as a structural component of membrane lipids and precursor of diverse antimicrobial compounds (Mahmud et al. 2002; Bode et al. 2006; Yamauchi 2010). Additionally, enzymes involved in the L-leucine catabolism have other biotechnological applications; for example, leucine dehydrogenase is used to produce tumor-suppressing gents and anti-HIV protease inhibitors (Yan and Liao 2009; Li et al. 2014).

The aim of this work is to review the metabolic pathways related to the L-leucine metabolism, that generate metabolites of biological or biotechnological interest, showing the potential of the L-leucine-derived compounds as commodity chemicals.

1.1 L-Leucine availability and uptake

L-leucine is essential for protein synthesis (Kamekura et al. 1986; Dai et al. 2012). It is synthesized de novo by a metabolic pathway first appearing in all organisms' last common ancestor (Fondi et al. 2007), and may be recycled from intracellular proteins (Sussman and Gilvarg 1969; Chaloupka and Strnadová 1982; Gonzales and Robert-Baudouy 1996) or may be drawn from the environment, as a product of the decomposition of organic matter that generates free amino acids, peptides and proteins (Coffin 1989; Baena et al. 1998a, b; Vinolas et al. 2001; Jones et al. 2004; Zhou et al. 2009). Microorganisms can thrive in almost any ecosystem (Baena et al. 1998a, b), even under extreme conditions, in oligotrophic environments (Coffin 1989; Nemecek-Marshall et al. 1995; Vinolas et al. 2001; Fütterer et al. 2004; Alam et al. 2005; Zhou et al. 2009), or those generated by human activities such as the sludge and wastewaters from agricultural and livestock activities (Friedrich and Antranikian 1996; Ramsay and Pullammanappallil 2001). This is due to a large number of bacteria being able to use L-leucine, along with other amino acids, as an alternative source of carbon and nitrogen (Eitinger et al. 2011). For this purpose, L-leucine and other amino acids are recycled from proteins by intracellular and extracellular proteases, rendering small peptides which are further processed by aminopeptidases into free amino acids that can be directly assimilated by microorganisms (Gonzales and Robert-Baudouy 1996; Ramsay and Pullammanappallil 2001; Gupta et al. 2002; van Kranenburg et al. 2002; Jones et al. 2004; Berggren et al. 2010).

L-leucine (as a free amino acid or as part of small peptides) enters bacterial cells through specific uptake transport systems whose coding sequences are highly conserved and distributed in a wide variety of bacterial genomes (Paulsen et al. 2000; Day et al. 2001; Takami et al. 2002; van Kranenburg et al. 2002; Johnson et al. 2008; Pletzer et al. 2014) such as the high-affinity branched-chain amino acid transporter (LIV-I) of the HAAT family (Dassa and Bouige 2001; Hosie and Poole 2001), the low-affintity branched-chain transporter (LIV-II) and the small peptide permeases (Dpp/

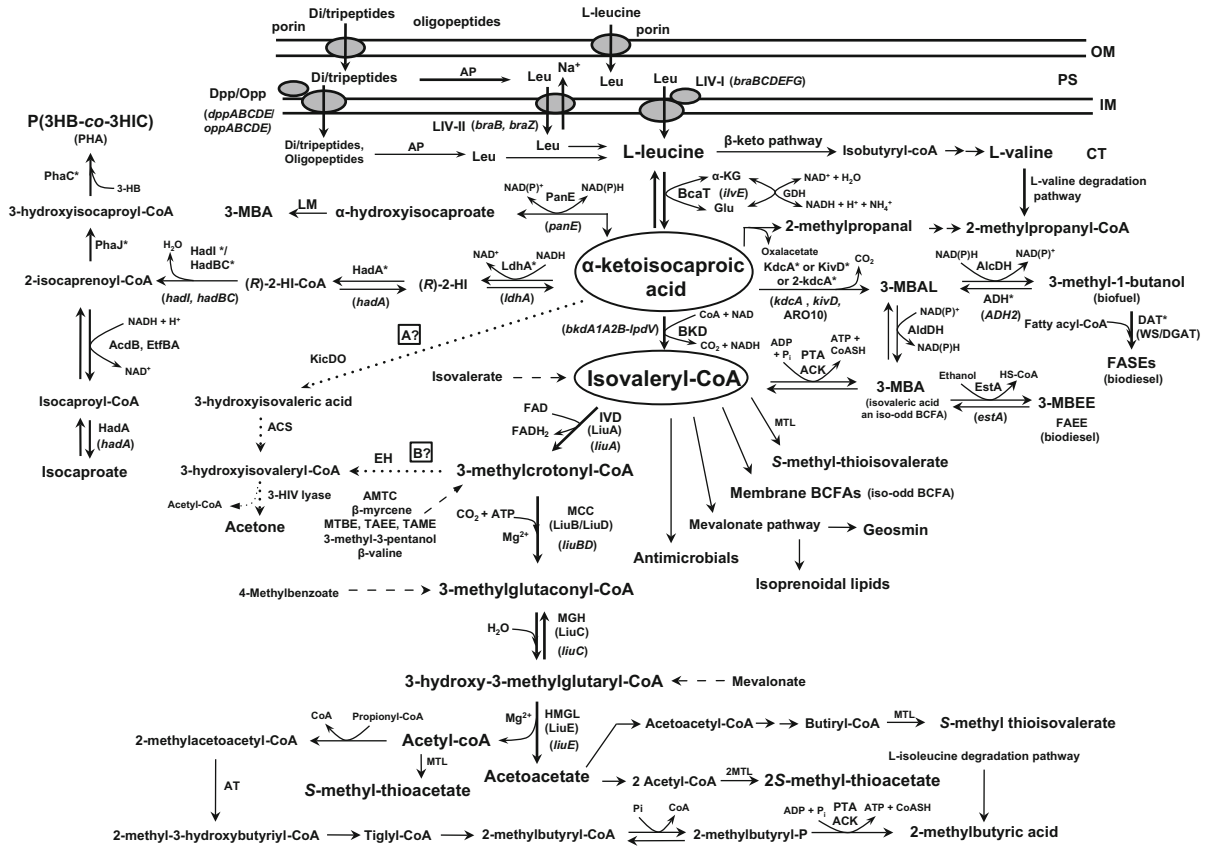

Fig. 1 Bacterial L-leucine metabolism as a source of secondary metabolites. The *diagram* shows the pathways related to the L-leucine catabolism; some steps are simplified and may be carried out by several enzymes acting sequentially. The cell envelope is represented at the *top* (*OM* outer membrane, *PS* periplasmic space, *IM* inner membrane, *CT* cytoplasm) and includes the L-leucine transporters (LIV-I, high-affinity branched-chain amino acid transporter; LIV-II, low-affinity branched-chain amino acid transporter; Dpp/Opp, di, tri/oligopeptide transporter). The L-leucine conventional catabolic pathway from *P. aeruginosa* and final products of alternative pathways of L-leucine catabolism are shown in *upper case letters*. The main intermediates that are derived through other pathways are shown in ovals. *Dashed arrows* indicate metabolites that converge with the L-leucine conventional pathway. *Dotted arrows* indicate uncertainty. *Asterisk* denotes enzymes used in engineered pathways in transgenic microorganisms that are used to produce biofuels and polyhydroxyalkanoates. Genes that have been characterized in the pathway are in parenthesis. Compounds and enzymes are as follows: *3-MBAL* 3-methylbutanal, *3-MBA* 3-methylbutanoic acid, *3MBEE* 3-methylbutanoate-ethyl ester, *(R)-2-HI (R)*-2-hydroxyisoaproate, *P(3HB-co-3HIC)* poly(3-hydroxybutyrate copolymer 3-hydroxyisocaproate, *PHA* polyhydroxyalkanoates, *BCFA* branched-chain fatty acid, *AMTC* acyclic monoterpenes of the citronellol family, *MTBE* methyl-*tert*-butyl ether, *TAEE* *tert*-amyl-ethyl ether, *TAME tert*-amyl methyl ether, *α-KG* α-ketoglutarate, *Glu* glutamate, *MTL* methanethiol, *AP* aminopeptidase, *GDH* glutamate dehydrogenase, *BcaT* branched-chain aminotransferase, *BKD* branched-chain α-ketoacid dehydrogenase complex, *IVD* isovaleryl-CoA dehydrogenase, *MCC* 3-methylcrotonyl-CoA carboxylase, *MGH* 3-methylglutaconyl-CoA hydratase, *HMGL* 3-hydroxy-3-methylglutaryl-CoA lyase, *KdcA* α-ketoacid decarboxylase from *L. lactis* B1157, *KivD* keto-isovaleric decarboxylase from *L. lactis* IFPL730, *2-kdcA* α-ketoacid decarboxylase from *S. cerevisiae*, *AlcDH* alcohol dehydrogenase, *AldDH* aldehyde dehydrogenase, *ADH* ADH from *S. cerevisiae, DAT* diacylglycerol acyltransferase from *A. baylyi* ADP1, *PhaC* polyhydroxyalkanoates synthase, *PhaJ (R)*-specific enoyl-CoA hydratase, *LdhA (R)*-2-hydroxy-isocaproyl dehydrogenase, *HadA (R)*-2-hydroxy-isocaproyl transferase, *HadBC (R)*-2-hydroxy-isocaproyl-CoA dehydratase, *Hadl* activator for dehydratase HadBC, *AcdB* acyl-CoA dehydrogenase, *EtfBA* electron transferring flavoprotein, *PanE* hydroxyl-isocaproate dehydrogenase, *LM* lactate mono-oxigenase, *EH* enoyl-CoA hydratase, *KicDO* keto-isocaproate dioxygenase, *ACS* acyl-CoA synthetase, *HIVL* 3-hydroxyisovaleryl-CoA lyase, *PTA* phosphotransferase, *ACK* acyl kinase, *EstA* esterase A, *AT* acyltransferase

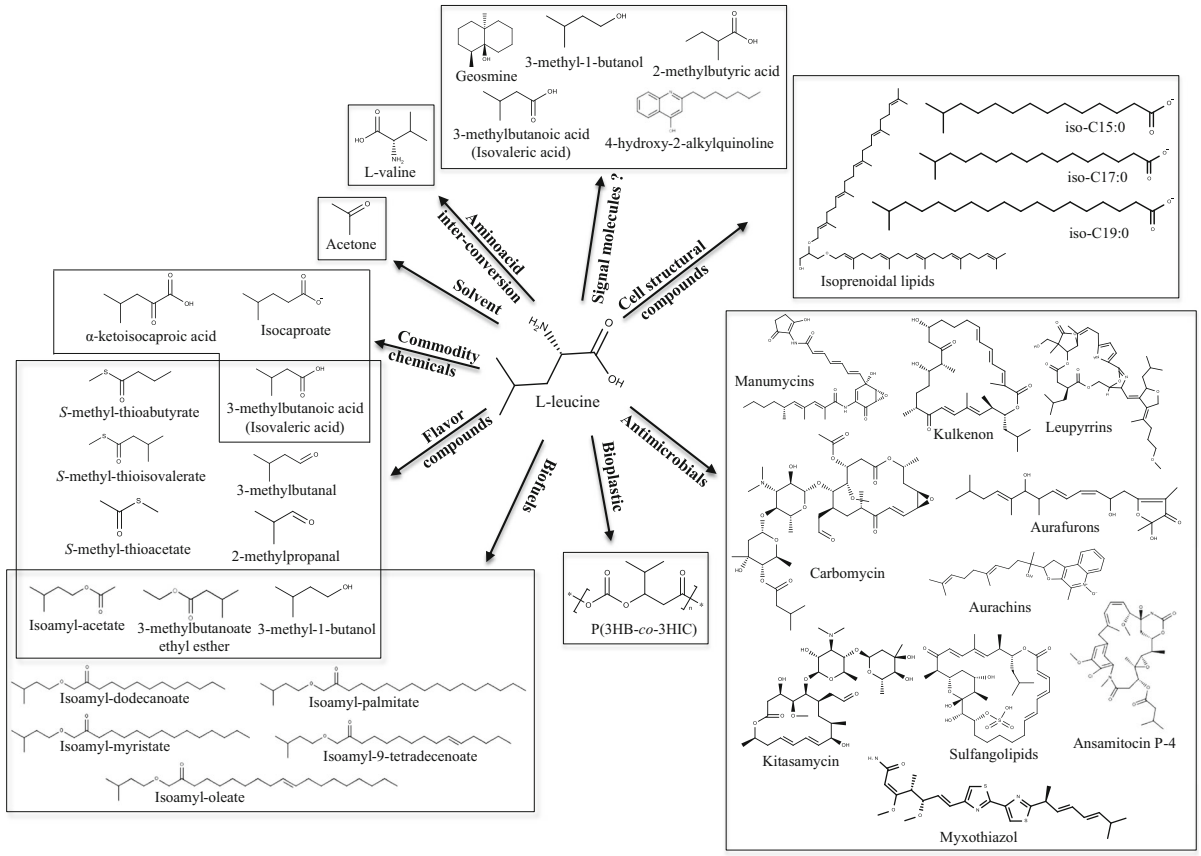

Fig. 2 Compounds of biotechnological importance obtained from L-leucine metabolism. The *boxed regions* depict the related biological function or biotechnological applications

Opp) of the PepT family (van Kranenburg et al. 2002; Eitinger et al. 2011; Sinz and Schwab 2012). LIV-I and Dpp/Opp are ABC transporters that depend of binding proteins. The structural genes of these transporters are commonly located in gene clusters and constitute a sole transcriptional unit but in some cases the gene that encodes the binding proteins are transcribed independetly or are located outside of the gene cluster (Hoshino and Kose 1990; Matsubara et al. 1992; Hosie and Poole 2001; Hosie et al. 2002; Eitinger et al. 2011). The binding proteins of the LIV-I and Dpp/Opp are capable of recognizing several substrates with differential specificities (Matsubara et al. 1992; Hosie et al. 2002; Eitinger et al. 2011) and, furthermore, the LIV-I and Dpp/Opp transporters can interact with more than one binding protein (Eitinger et al. 2011; Pletzer et al. 2014). The binding proteins of the LIV-I transporter belongs to a family of binding proteins that are specific for aliphatic amino acids and amides (Tam and Saier 1993); these proteins are periplasmic in Gram-negative bacteria (Ohnishi and Kirttani 1983), whereas in Gram-positive bacteria and archea, the proteins are attached to the cell membrane (Hosie et al. 2002).

The LIV-II, along with non-specific porins, transport L-leucine with low affinity binding. LIV-II is a protein composed of 12 membrane-spanning segments, whose activity is coupled to an electrochemical potential of protons or monovalent ions (Hoshino et al. 1990, 1991; Stucky et al. 1995; Vijaranakul et al. 1998).

These systems represent the main routes in L-leucine uptake (Matsubara et al. 1992; Day et al. 2001) and play important roles in the nitrogen intake of heterotroph bacteria capable of amino acids assimilation, such as those from the *Clostridium* genus, and species *Idiomarina loihiensis*, *Bdellovibrio bacteriovorus*, and *Oceanobacillus iheyensis*

(Takami et al. 2002; Hou et al. 2004; Barabote et al. 2007; Sebaihia et al. 2007; Pletzer et al. 2014) and in amino acids auxotropic bacteria, such as lactic acid bacteria (LAB) (van Kranenburg et al. 2002; Pletzer et al. 2014; Smid and Kleerebezem 2014). Interestingly, L-leucine transporters have been related to phenomena such as colonization (Mueller 1996; Ahn et al. 2007), virulence (Basavanna et al. 2009; Eitinger et al. 2011), signaling (Eitinger et al. 2011), systemic resistance (Han et al. 2006), alkaline adaptation (Takami et al. 2002), halotolerance (Hoshino et al. 1991), survival under extreme conditions such as low temperatures (Fonseca et al. 2011), acidophilic (Fütterer et al. 2004) and nitrogen limiting environments (Nikodinovic-Runic et al. 2009), utilization of aromatic compounds (Zhao et al. 2005; Kurbatov et al. 2006; Yun et al. 2011), and adaptation to environments where proteins are the major compounds, such as that of *P. aeruginosa* in the lungs of patients with cystic fibrosis (CF) (Hoboth et al. 2009). It is clear that expression of the L-leucine transporters seems to be a general mechanism of carbon utilization and starvation response. Transporters are essential features that enable co-utilization of nutrients, and the amino acids transport represents an adaptative mechanism that responds to the continuosly changing availability of nutrients, given that their levels in most environments rarely offer sufficient amounts for growth-saturation of microbial communities (De Mattos and Neijssel 1997; Silberbach et al. 2005). Cell transport sustains growth is an ability that cells maintain for growing when nutrients become unavailable in environment (Siegele and Kolter 1992; Cao and Loh 2008). However, this ability is subjected to strict regulation mechanisms that modulate expression of genes involved in L-leucine uptake, which depends on the L-leucine availability through Lrp-mediated repression (Antonucci et al. 1986; Haney et al. 1992; Calvo and Matthews 1994; Belitsky et al. 1997; Ohnishi et al. 1999; Johnson et al. 2008; Scully et al. 2015). In addition, L-leucine uptake is also subjected to the post-transcriptional regulation system CbrAB/Crc (carbon catabolite control system) (Kurbatov et al. 2006; Moreno et al. 2009; Rojo 2010; Sonnleitner et al. 2012; Fonseca et al. 2013), involved in assimilation of preferential carbon and energy sources in which carbohydrates are preferred over amino acids (Rojo 2010).

1.2 L-Leucine conventional catabolic pathway

L-Leucine catabolism occurs frequently in bacteria (Ganesan et al. 2006; Kazakov et al. 2009) and has been widely studied in the *Pseudomonas* genus (Massey et al. 1974, 1976; Diaz-Perez et al. 2004; Aguilar et al. 2006; Förster-Fromme and Jendrossek 2006; Chávez-Avilés et al. 2009). The catabolism of the branched-chain amino acids (L-leucine, L-isoleucine, and L-valine; BCAA) was first described in bacteria in the 1970s (Massey et al. 1976). It can be divided into two stages: (1) the common pathway, which is comprised by the initial three reactions, shares enzymes with isovalerate assimilation and is encoded by several gene clusters, and (2) the distal pathway (LIU pathway) which is also composed by three reactions and is specific for L-leucine catabolism. In the past decade, the *liuR-ABCDE* cluster that encodes the distal pathway was identified in *P. aeruginosa*, and some of its enzymes were characterized (Campos-García 2010; Förster-Fromme and Jendrossek 2010). Interestingly, this gene cluster was not found in *Escherichia coli* (Kazakov et al. 2009); in fact, L-leucine was demonstrated to be repellent of chemotaxis in these bacteria, enabling them to avoid high concentrations of L-leucine, since it can inhibit cell growth by affecting the biosynthesis of other amino acids (Yang et al. 2015).

The L-leucine deamination is the first reaction of the common pathway and renders α-ketoisocaproic acid (Fig. 1), which serves as a backbone for the synthesis of metabolites of biotechnological interest in fuel and food industries (Afzal et al. 2013; Rabinovitch-Deere et al. 2013; Saika et al. 2014), spanning an ample variety of applications in the medical and biology areas (Vogt et al. 2015). This reaction is conducted by common enzymes for BCAA, generally occurring via one of two enzymatic reactions: (1) oxidative deamination, catalyzed by L-amino acid oxidase and leucine dehydrogenase (LDH); or (2) transamination, catalyzed by aminotransferases, that in the presence of pyridoxal-5-phosphate, transfer the amino group of the amino acid to an α-keto acid acceptor, preferentially α-ketoglutarate. The α-keto acids derived from L-valine and L-leucine, along with pyruvate and oxalacetate, work as amino group acceptors (Massey et al. 1976; Larrouture et al. 2000; Beck et al. 2004; Ziadi et al. 2010; Freiding et al. 2012).

Due to the biotechnological importance of L-leucine deamination, it has been thoroughly characterized (Massey et al. 1976; Kuramitsu et al. 1985; Katoh et al. 2003; Mahdizadehdehosta et al. 2013; Li et al. 2014). The reaction is carried out by transaminase B from *P. aeruginosa* (Massey et al. 1976) and *Corynebacterium glutamicum*, the major host for biotechnological production of amino acids (Vogt et al. 2015) or by the branched-chain amino acid aminotransferase (BcaT), encoded by the *ilvE* gene (Massey et al. 1976; Kuramitsu et al. 1985; Ziadi et al. 2010; Sinz and Schwab 2012), and by the aromatic amino acid aminotransferase (AraT) and the aspartate aminotransferase (AspT) (Ziadi et al. 2010; Freiding et al. 2011, 2012). In LAB and bacteria that have the flavor-forming pathway, L-leucine deamination is only performed by aminotransferases (Larrouture et al. 2000; Beck et al. 2004; Freiding et al. 2012), whereas in *Rhodococcus opacus* DSM 43250, L-leucine deamination is catalized by L-amino acid oxidase, which has been utilized to produce α-ketoisocaproic acid with L-leucine as its substrate (Zhu et al. 2011).

The second reaction of the common pathway comprises the oxidative decarboxylation of the α-ketoisocaproic acid to isovaleryl-CoA (Fig. 1). The isovaleryl-CoA intermediary is particularly important in the pathway, because the isovalerate catabolism converges at this point through the activation of isovaleric acid by an acyl-CoA synthetase (Watkins 1997; Campos-García 2010; Förster-Fromme and Jendrossek 2010). Isovaleryl-CoA can be deviated to feed other metabolic pathways that may be used to produce important metabolites for the cell, such as branched-chain fatty acids (BCFA) (Kaneda 1991; Mahmud et al. 2002; Thierry et al. 2002; Bode et al. 2005). The reaction is carried out by a common, widely studied branched-chain α-keto acid dehydrogenase complex (BKD), encoded by the *bkdA1A2B-lpdV* gene cluster (Massey et al. 1976; Sokatch et al. 1981; Denoya et al. 1995; Thierry et al. 2002; Bode et al. 2006; Sonnleitner et al. 2012). The isovaleryl-CoA is dehydrogenated by the isovaleryl-CoA dehydrogenase (LiuA) encoded by the *liuA* gene, rendering 3-methylcrotonyl-CoA (Förster-Fromme and Jendrossek 2008). The acyclic terpene utilization (ATU) pathway, which is involved in the degradation of acyclic monoterpenes of the citronellol family (AMTC) (Aguilar et al. 2006), converges at this point, as well as the β-myrcene catabolism in *Pseudomonas* sp. M1, which involves its transformation into geraniol and geranic acid. β-myrcene induces the expression of the *atu* and *liu* gene clusters (Soares-Castro and Santos 2015). Other compounds that converge with the 3-methylcrotonyl-CoA intermediate are methyl *tert*-butyl ether (MTBE), *tert*-amyl-methyl ether (TAME) and *tert*-amyl-ethyl ether (TAEE), which are used as gasoline additives, and are also considered important pollutants. The degradation of MTBE, TAME, TAEE, and the tertiary alcohol 3-methyl-3-pentanol in the bacteria *Aquincola tertiaricarbonis* L108 and *Methylibium petroleiphilum* PM1, produce 3-methylcrotonyl-CoA as intermediate (Schuster et al. 2012, Hyman 2013). Another catabolic pathway that has been recently found to converge with the 3-methylcrotonyl-CoA is the β-valine catabolic pathway from *Pseudomonas* sp. SBV1 through the β-valinyl-CoA intermediate (Otzen et al. 2015), which indicates that the metabolism of these compounds are linked to the L-leucine catabolism.

The initial reaction in the distal pathway of L-leucine catabolism involves the carboxylation of 3-methylcrotonyl-CoA to produce 3-methylglutaconyl-CoA. The 4-methylbenzoate degradation pathway from *Magnetospirillum* sp. pMbN1 converges at this point (Lahme et al. 2012). The carboxylation reaction is performed by the 3-methylcrotonyl-CoA carboxylase, conformed by the LiuB and LiuD subunits which are encoded by the *liuB* and *liuD* genes, respectively (Massey et al. 1976; Aguilar et al. 2008; Campos-García 2010). In prokaryotes, the 3-methylcrotonyl-CoA carboxylase has only been structurally characterized in *P. aeruginosa* and *Mycobacterium tuberculosis* (Ehebauer et al. 2015). This type of carboxylase is classified as an "assimilatory carboxylase" because the incorporation of CO_2 by the enzyme into the substrate allows for the formation of a functional group, enabling further transformation of the substrate into a central precursor molecule (Erb 2011). That reaction could be of great importance for the metabolism of bacteria in resources-depleted media since it confers an alternative route to assimilate carbon from an inorganic source when nutrients are scarce (Ganesan et al. 2006; Alonso-Sáez et al. 2010).

In the next reaction, the 3-methylglutaconyl-CoA is converted into 3-hydroxy-3-methylglutaryl-CoA by the 3-methylglutaconyl-CoA hydratase. It has been suggested that in *P. aeruginosa* the *liuC* gene could encode that enzyme. The 3-methylglutaconyl-CoA

enzyme from *Acinetobacter* sp. IVS-B is an homotetramer, independent of any cofactor, that has been shown to catalyze the hydration of 3-methylglutaconyl-CoA and dehydration of 3-hydroxy-3-methylglutaryl-CoA as their straight-chain derivatives (Mack et al. 2006). Notably, the mevalonate degradation pathway converges at this point (Massey et al. 1976).

The final reaction of the distal L-leucine assimilation pathway is carried out by the bifunctional enzyme LiuE, encoded by the *liuE* gene (Chávez-Avilés et al. 2009, 2010). This enzyme is capable of using both the 3-hydroxy-3-methylglutaryl-CoA (from L-leucine catabolism) and the 3-hydroxy-3-isohexenylglutaryl-CoA (from the ATU pathway) as substrates. LiuE is a 3-hydroxy-3-methylglutaryl-CoA lyase that cleaves the 3-hydroxy-3-methylglutaryl-CoA to render acetyl-CoA and acetoacetate; these products can be then channeled to the tricarboxylic acids cycle and the glyoxylate shunted to synthesize other biomolecules when the organic precursors do not satisfy cellular requirements (Díaz-Pérez et al. 2007; Alonso-Sáez et al. 2010; Scoffield 2012).

L-Leucine catabolism is intensified under low-nutrients conditions, such as those occurring in starvation and oligotrophic environments (Alonso-Sáez et al. 2010); however, in strains, such as the phytopathogen *Pseudomonas syringae* species, the L-leucine catabolism is important but not essential for bacterial survival under low L-leucine availability in environment (Rico and Preston 2008).

1.3 Regulation of L-leucine assimilation

The modulation of the L-leucine metabolism in bacteria has been studied in detail in the *Pseudomonas* genus. The regulation of L-leucine catabolism is controlled at different levels. One of them is at the transcriptional level, such as the BkdR regulator involved in positive control of the *bkdA1A2B-lpdV* gene cluster and the LiuR encoded by the first gene of the *liuRABCDE* gene cluster. In gamma and beta-proteobacteria it has been proposed that the genes of the LiuR regulon are implicated in the catabolism of BCAA (Kazakov et al. 2009). The L-leucine catabolism also depends on sigma factors; for example, in *Pseudomonas fluorescens* SBW25, a mutation in *rpoN* (σ^{54}) produces a clear impairment in the ability to use L-leucine as the sole source of carbon and nitrogen (Jones et al. 2007). Another level of regulation is through the CbrAB/Crc system, which represses the *ilvE* gene and the *liuABCD* gene cluster (Linares et al. 2010; Fonseca et al. 2011, 2013). The *bkdA1A2B-lpdV* gene cluster is positively regulated by the transcriptional regulator BkdR, which is repressed by the CbrAB/Crc system (Massey et al. 1974; Moreno et al. 2009; Fonseca et al. 2011; Sonnleitner et al. 2012; Fonseca et al. 2013). During cold adaptation the *bkdA1A2B-lpdV* and *liuABCD* gene clusters are upregulated as the isovaleryl-CoA, an intermediate of the pathway, is used as a precursor of BCFA synthesis. This is important for maintaining membrane fluidity during temperature changes or compensating lower catalytic activity of susceptible enzymes (Fonseca et al. 2011, 2013; Frank et al. 2011). Interestingly, in *Pseudomonas brassicacearum*, the *ldh* expression responds to cadmium (Sanchez et al. 2007), whereas in *P. aeruginosa*, *ldh* is upregulated in mature biofilm structure (Vilain et al. 2004) and is positively co-regulated by the stationary-phase sigma factor σ^{s} (RpoS) and quorum sensing (Schuster et al. 2004; Juhas et al. 2005). In the stationary phase, the *bkdA1A2B-lpdV* and *liuABCD* gene clusters are upregulated (Waite et al. 2006), thus activating L-leucine catabolism in this phase, characterized by a lack of nutrients and increased protein turnover (Siegele and Kolter 1992; Silberbach et al. 2005). In summary, due to its abundance in proteins, L-leucine represents an important source of energy, carbon, and nitrogen for bacterial metabolism maintenance (Brinkman et al. 2003; Ganesan et al. 2006; Díaz-Pérez et al. 2007). The systems that regulate the L-leucine degradation pathway must be induced by initial substrates or intermediates that converge in the pathway. For example, the *bkdA1A2B-lpdV* gene cluster is induced by BCAA and branched-chain keto acids (Massey et al. 1974). Also, the *liuABCD* gene cluster is induced by isovalerate, 3-methylcrotonate, citronellol, and L-leucine (Massey et al. 1974; Aguilar et al. 2006).

L-Leucine catabolism is highly repressed by the existence of other preferential substrates posing as good carbon sources such as succinate or glucose (Rojo 2010); however, the L-leucine catabolism is important in starvation conditions. Bacteria in the environment predominantly exist in sessile communities, rather than as free-living cells, and develop biofilms (Vasseur et al. 2005) which are involved in bacterial infections (Attila et al. 2008). In this lifestyle,

the *bkdA1A2B-lpdV* and *liuABCD* gene clusters are upregulated in early biofilm formation (Waite et al. 2006), but are downregulated in mature biofilms (Attila et al. 2008; Patell et al. 2010), indicating that these gene clusters could be important for the dynamics of the biofilms. The L-leucine catabolism has also been reported in humans with CF. *Pseudomonas aeruginosa* strains isolated from patients with CF showed an increased transcription of the *liuABCD* gene cluster (Klockgether et al. 2013), while in *P. aeruginosa* C, a CF lung-adapted strain, the enzymes BraC and BKD involved in the metabolism of L-leucine were downregulated (Sriramulu et al. 2005), even though L-leucine is abundant in CF sputum (Palmer et al. 2007; Patell et al. 2010; Sonnleitner et al. 2012). This suggests, suggesting that multiple pathways are present as mechanisms, including L-leucine degradation pathway may directly be associated with the establishment of chronic infections in the lungs of patients with CF (Huse et al. 2010).

1.4 Relation of L-leucine assimilation with the environmental adaptation of microorganisms

The L-leucine uptake and assimilation in bacteria play important roles in bacterial adaptation (Eitinger et al. 2011; Pletzer et al. 2014) since the conventional L-leucine assimilation pathway provides compounds that can enter routes of central metabolism for precursor synthesis; therefore, bacteria can proliferate in low-nutrient conditions or in protein-rich environments (Coffin 1989; Vinolas et al. 2001). However, alternate catabolism of L-leucine generates compounds that, apart from being to ATP production, may play a different physiological role in both carbon and nitrogen assimilation. This phenomenon has been reported at the onset of the stationary phase in *Lactococcus lactis*, in which it begins to use proteins for energy extraction. Further incubation results in a non-culturable state, where cells remain for long periods of time (at least 4 years) since homeostatic conditions are met. In this stage they redirect and reuse cofactors for carbon assimilation (Ganesan et al. 2006). During this time period, sugar reserves are depleted, and alternative sources of energy, such as amino acids, are required to maintain metabolic activity (Siegele and Kolter 1992). This mechanism allows for the survival of the cell during starvation stress. *L. lactis,* during prolonged starvation periods, produces 3-methyl-butanoic acid (3-MBA) and 2-methyl-butanoic acid (2-MBA) from L-leucine catabolism (Ganesan et al. 2006; Serrazanetti et al. 2011). *Propionibacterium freudenreichii* produces 3-MBA during prolonged starvation (Dherbecourt et al. 2008), whilst *Staphylococcus xylosus* triggers the synthesis of 3-MBA from L-leucine at stationary phase (Beck et al. 2004). Contrarily, the exposure to stress by acids in *Lactobacillus sanfranciscensis* increases the production of 3-MBA and 2-MBA during growth with fructose or maltose, generating three mol of ATP per mol of L-leucine by synthesizing 2-MBA (Ganesan et al. 2006; Serrazanetti et al. 2011). In fact, the exposure of microbial species to osmotic, acid, oxidative, and chemical stresses gives rise to an accumulation (20–30-fold) of 3-MBA generated from L-leucine catabolism (Serrazanetti et al. 2011).

On the other hand, in the cyanobacteria *Microcystis aeruginosa*, the production of 3-methyl-1-butanol from L-leucine coincides with the death phase (Hasegawa et al. 2012). In *Saccharomyces cerevisiae* 3-methyl-1-butanol induces pseudohyphal growth, indicating a quorum-signaling pathway that triggers morphologic changes (Hazelwood et al. 2008; Hasegawa et al. 2012). The over-production of 3-MBA, 2-MBA, and 3-methyl-1-butanol suggests that those compounds may act as signal molecules for switching from sugar to amino acid catabolism, supporting growth in restricted environments or related to the lifecycle of the microorganism. In recent years, it has been found that the compounds produced by this microorganism act as newly described signal molecules. For example, the cyclic dipeptide cyclo-(L-Pro-Leu) acts as an exogenous auto-inducer that in *Shewanella putrefaciens* and *Shewanella baltica* regulates the LuxR expression, the production of biofilm matrixes, extracellular proteases, and increases the bacterial growth rates and population (Zhu et al. 2015). In diatoms, polyunsatured short-chain aldehydes are produced, which are involved in intercellular communication and interactions with competitors (Leflaive and Ten-Hage 2009). Therefore, it is possible that metabolites from L-leucine pathways such as 3-MBA, 2-MBA, and 3-methyl-1-butanol could function as signal molecules; however, it is unclear if those molecules are used as carbon and energy sources or if their function is associated to molecular signals that switch metabolism or trigger the life cycle stages of the bacteria.

2 Biotechnological potential of the L-leucine catabolism

Nowadays depletion of fossil fuels, which are basic on the chemical synthesis, has led to the search of new commodity chemicals from renewable, cleaner, and relatively cheaper raw material (Rodriguez and Atsumi 2012; Varman et al. 2013). Therefore, bacterial metabolism has become a promising source of molecular structures that have the potential to be used as raw chemicals. For this purpose, the L-leucine catabolism provides a reservoir of metabolic intermediates which can be used as precursor molecules to synthesize products of biotechnological interest (Gutsche et al. 2012; Saika et al. 2014; Vogt et al. 2015). The potential of L-leucine as a renewable resource is supported by the fact that it is not only assimilated by bacteria through the L-leucine conventional catabolic pathway but it, since in different bacterial *phyla*, this amino acid is diverted to others routes in different bacterial genus, generating intermediates or final products that can be considered commodity chemicals or might be used directly for biotechnological applications (Nemecek-Marshall et al. 1999; Kim 2004; Gutsche et al. 2012). For example, in *Firmicutes,* the final products of L-leucine catabolism comprise higher alcohols, such as 3-methyl-1-butanol or its oxidized or reduced products, which are related with the flavor and aroma in food. On the other hand, research has demonstrated that 3-methyl-1-butanol has the biotechnological potential as biofuel (Connor and Liao 2008). Other intermediates of the L-leucine catabolism are also of interest, as will be seen below.

2.1 Inter-conversion of metabolic pathways branched chain amino acids assimilation

2.1.1 Inter-conversion of the L-leucine and L-isoleucine pathways

The inter-conversion between L-leucine and L-isoleucine metabolisms is carried out via a 2-methylbutyric acid intermediate, which is produced from L-leucine catabolism. It is also an intermediate metabolite of the L-isoleucine degradation pathway; thus, an inter-conversion exists between both catabolic pathways (Liu et al. 2014). The production of 2-methylbutyric acid from L-leucine has been shown in several strains, such as *Lactobacillus* spp., *L. sanfranciscensis* LCE1, *L. lactis* IL403, and *Clostridium* spp. (Ganesan et al. 2006; Serrazanetti et al. 2011). *L. lactis* IL1403 has the ability to transform L-leucine into branched-chain fatty acids to BCFA, during a nonculturable state induced by sugar starvation, producing 2-methylbutyric acid rather than isovaleric acid, which is the expected product from the L-leucine catabolism (Thierry et al. 2002; Ganesan et al. 2006; Dherbecourt et al. 2008). In this pathway, L-leucine is assimilated through its conventional catabolic pathway up until the formation of acetoacetyl-CoA and acetyl-CoA. Subsequently, acetyl-CoA reacts with propionyl-CoA in order to produce 2-methyl-3-hydroxybutyryl-CoA (Fig. 1). The proposed enzyme for performing this reaction is acyltransferase.

It has been hypothesized that forward in the pathway, activities of a phosphotransacylase, along with an acyl-kinase, generates 2-methylbutyric acid plus ATP via substrate-level phosphorylation (Fig. 1). Theoretically, the L-leucine transformation into 2-methylbutyric acid requires one ATP as investment to generate one molecule of acetyl-CoA and one of acetoacetyl-CoA, whose cleavage produces two additional molecules of acetyl-CoA; eventually, each acetyl-CoA reacts with propionyl-CoA to produce three molecules of 2-methylbutyric acid and three additional ATP molecules (Ganesan et al. 2006).

2.1.2 Inter-conversion of the L-leucine and L-valine pathways

Similarly to the inter-conversion pathway between L-leucine and L-isoleucine, the L-leucine catabolism is connected to the L-valine catabolic pathway by the 2-methyl-propanal intermediate (Fig. 1). The production of 2-methyl-propanal in *L. lactis* B1157 does not occur through an enzymatic process, as it was demonstrated that the α-ketoisocaproic acid is chemically converted into 2-methyl-propanal (Smit et al. 2004a, b, 2009; Liu et al. 2014). This inter-conversion pathway was also predicted in silico as part of the bacterial flavor-forming metabolism (Liu et al. 2014). In this inter-conversion pathway, the α-ketoisocaproic acid originated from L-leucine reacts with oxygen, producing 2-methyl-propanal and oxaloacetate. This chemical reaction depends on the manganese concentration, presence of oxygen, and redox potential (Smit et al. 2004a, b, 2009). The 2-methyl-propanal is then

oxidized by an aldehyde dehydrogenase, rendering 2-methyl-propanoic acid, which may be activated by an acyl-CoA synthetase to obtain 2-methyl-propionyl-CoA intermediary, also produced during the L-valine catabolism (Massey et al. 1976). The L-leucine-L-valine inter-conversion in Clostridia involves the β-keto pathway, which converts L-leucine into isobutyrate (Fig. 1) (Allison and Peel 1971; Poston 1976; Monticello and Costilow 1982). The β-keto pathway was postulated by Poston (1976) in *Clostridium sporogenes,* where α-L-leucine is isomerized to β-L-leucine via a reversible reaction carried out by the enzyme leucine 2,3-aminomutase (Poston 1976; Barker 1981; Poston 1986). This reaction is a rate-limiting step; therefore, it is key in the biosynthesis of isobutyrate. This enzyme uses adenosylcobalamin (coenzyme B_{12}) as cofactor (Poston 1976, 1984, 1986). Another aminomutase, β-lysine 5,6-aminomutase uses adenosylcobalamin instead of S-adenosyl-L-methionine to generate a 5′-deoxyadenosyl radical, which causes a similar reaction to rearranging the 6-amino group to the C5 position, producing 3,5-diaminohexanoic acid. It is likely that leucine 2,3-aminomutase carries out a similar mechanism (Poston 1986; Kudo et al. 2014). β-ketoisocaproic acid may result from the transamination of β-amino group on the backbone, likely by a ω-aminotransferase. Many of the substrates of these special aminotranferases are amino acids whose amino group is located away from the carboxyl group; however, there are ω-aminotransferases whose substrates have the amino group at C2 of their backbone; for example, AptA from *Alcaligenes denitrificans* Y2k2 catabolizes the transamination of β-L-leucine using α-ketoglutarate as amino group acceptor (Shin et al. 2003; Yun et al. 2004; Rausch et al. 2013).

The next step of the β-ketopathway is the activation of β-ketoisocaproic acid by a Coenzyme A transferase or an acyl-CoA synthetase, producing β-ketoisocaproil-CoA, which by a thiolase activity, renders isobutyryl-CoA and acetyl-CoA as reaction products (Poston 1986). This pathway is biologically feasible since L-valine is synthesized from isobutyryl-CoA. Such metabolite should be channeled to L-valine synthesis, where isobutyryl-CoA is carboxylated to α-ketoisovalerate. Finally, by the transference of its amino group, L-valine may be produced (Allison and Peel 1971; Monticello and Costilow 1982; Liu et al. 2014).

2.2 Products obtained from oxidoreduction of L-leucine by α-ketoisocaproic acid catabolism

Anaerobic bacteria use L-leucine as an energy source in the presence of conventional final electron acceptors, such as oxygen, nitrate, sulfate, acetate, crotonate, and even hydrogen-using bacteria, such as methanogens, acetogenic bacteria, or sulfate-reducing bacteria that can receive electrons as shown in Table 1 (Fardeau et al. 1997; Baena et al. 1998a, b; Ramsay and Pullammanappallil 2001; Menes and Muxí 2002; Kim et al. 2006; Sallam and Steinbüchel 2009; Ato et al. 2014). During this process, L-leucine is oxidized through a conventional degradation pathway, which in anaerobic bacteria usually does not differ from L-leucine catabolism present in aerobic microorganisms (Kim 2004; Kim et al. 2005). In the anaerobic condition, the oxidative deamination is the first step in the L-leucine oxidation, which yields α-ketoisocaproic acid. This step is endergonic (Table 2, Eq. 1) and proceeds only if the excess of released electrons is rapidly removed (Kim et al. 2005; Ato et al. 2014). This step of the pathway in *Prevotella intermedia* and *Prevotella nigrescens* is performed by the BcaT, not by LDH, and uses 2-ketoglutarate as the amino group acceptor. The decarboxylation of the α-ketoisocaproic acid produces isovaleryl-CoA. In the strains mentioned above and hyperthermophilic archeas, such as *Thermococcus litoralis*, *Thermococcus* sp. ES-1, *Pyrococcus furiosus,* and *Pyrococcus* sp. ES4, the reaction is realized by the branched-chain 2-oxoacid oxidoreductase enzyme instead of BKD, as occurs in *Pseudomonas* genus (Massey et al. 1976; Heider et al. 1996; Takahashi and Yamada 2000). The 2-oxoacid oxidoreductase is a dimer of heterotetramers ($\alpha_2\beta_2\gamma_2\delta_2$), with three [4Fe4S] clusters per heterotetramer (Afzal et al. 2013). The catalytic activity of this enzyme depends on the presence of thiamine pyrophosphate, CoA, Mg^{2+}, and methylviologen or ferredoxin as electron acceptors instead of NAD (Heider et al. 1996). Down on the pathway, isovaleryl-CoA is transformed to isovaleric acid by the activities of a phosphotransacylase and acyl kinase enzymes. This reaction also generates ATP via substrate-level phosphorylation (Fig. 1) (Barker 1981; Kim et al. 2005, 2006; Ato et al. 2014). In thermophilic strains from genera *Thermoanaerobacter* and *Caldanaerobacter*, besides from isovaleric acid, the corresponding branched-chain alcohol is

Table 1 Products with biotechnological importance obtained from bacteria L-leucine fermentation

Specie	Electron donor	Electron acceptor	Products	References
Desulfovibrio aminophilus sp.	L-Leucine	Sulfate	Isovalerate	Baena et al. (1998a, b)
Thermoanaerobacter brockii HTD4^{T}	L-Leucine	*Methanobacterium* sp. or thiosulfate	Isovalerate/H_2S	Fardeau et al. (1997)
T. brockii DSM 1457	L-Leucine	Thiosulfate	Isovalerate, 3-methyl-1-butanol/H_2S	Scully et al. (2015)
Anaeraculum mobile sp. nov.	L-Leucine	Crotonate	Isovalerate/butyrate	Menes and Muxí (2002)
Peptostreptococcus anaerobius	L-Leucine	L-Leucine	Isovalerate/isocaproate	Britz and Wilkinson (1982)
C. thiosulfatireducens DSM 13105^{T}	L-Leucine	Thiosulfate	Valerate/H_2S	Sallam and Steinbüchel (2009)
C. tunisiense DSM 15206	L-Leucine	Sulfur	Isovalerate/S^{-2}	Sallam and Steinbüchel (2009)
C. bifermentans	L-Leucine	L-Leucine	Isovalerate/isocaproate	Elsden and Hilton (1978)
Selenomonas acidaminovorans	L-Leucine	H^+ or an methanogen	Isovalerate H_2/CH_4	Guangsheng et al. (1992)

ND not determined

Table 2 Reactions and Gibbs free energy involved in the L-leucine fermentation processes

Reaction	Value of Gibbs free energy change	References
Equation 1: L-leucine + $2H_2O$ → $2H_2(4[H])$ + Isovalerate$^-$ + NH_4^+ + H^+ + HCO_3^- + ATP	$\Delta G^{o\prime} = +4.2$ kJ	Ramsay and Pullammanappallil (2001, Kim et al. (2005), Ato et al. (2014)
Equation 2: 2L-leucine + $2H_2(4[H])$ → 2Isocaproate + $2NH_4$		Kim et al. (2005)
Equation 3: 3L-leucine + $2H_2O$ → Isovalerate + 2Isocaproate + $3NH_4$ + HCO_3^- + ATP	$\Delta G^{o\prime} = -146.1$ kJ	Kim et al. (2005)
Equation 4: $2H_2(4[H])$ + 2Acetate$^-$ + H^+ → Butyrate$^-$ + $2H_2O$	$\Delta G^{o\prime} = -48.1$ kJ	Ato et al. (2014)
Equation 5: $3H_2(6[H])$ + 2Acetate$^-$ + H^+ + HCO_3^- → Propionate$^-$ + $3H_2O$	$\Delta G^{o\prime} = -76.1$ kJ	Ato et al. (2014)
Equation 6: $4H_2(8[H])$ + 2Acetate$^-$ + H^+ + $2HCO_3^-$ → Acetate$^-$ + $4H_2O$	$\Delta G^{o\prime} = -104.6$ kJ	Ato et al. (2014)
Equation 7: $10H_2(20[H])$ + $4HCO_3^-$ + $3H^+$ → Butyrate$^-$ + $10H_2O$	$\Delta G^{o\prime} = -257.3$ kJ	Ato et al. (2014)
Equation 8: 5L-leucine + $5H_2O$ → 5Isovalerate$^-$ + Butyrate$^-$ + $5NH_4$ + $2H^+$ + HCO_3^-	$\Delta G^{o\prime} = -236.3$ kJ	Ato et al. (2014)

obtained from L-leucine degradation as one of the final products in the presence of thiosulfate (Scully and Orlygsson 2014; Scully et al. 2015). In the *Clostridium* genus and other anaerobic bacteria, the absence of a final electron acceptor can favor L-leucine fermentation, since such microorganisms perform the Stickland reaction (Britz and Wilkinson 1982; Kim et al. 2005, 2006). This feature has been related with the

pathogenicity of *Clostridium difficile* (Jain et al. 2010). In the Stickland reaction, the oxidation of an amino acid is coupled with the reduction of other amino acids, and NAD/NADH is one of the redox carriers (Nisman 1954; Schwartz and Schäfer 1973). The oxidation of L-leucine (oxidative route) can be coupled to L-leucine reduction (reductive branch) (Kim et al. 2005, 2006; Ato et al. 2014). After L-leucine oxidation, this amino acid can be used as an electron donor, and the isovaleric acid is obtained as final product. Contrarily, when L-leucine acts as an electron acceptor under reduction reaction, the final product is isocaproic acid, which contains the same number of carbon atoms as the original amino acid (Barker 1981).

The pathway of the reductive route of L-leucine fermentation has been proposed in *C. difficile*, and the genes that encode the enzymes involved in this pathway constitute the *ldhAhadAIBCacdBetfB1A1* gene cluster (Kim et al. 2005, 2006; Sebaihia et al. 2007). In the first step of the reductive route of the L-leucine fermentation α-ketoisocaproic acid is produced, as in L-leucine oxidation, which is then reduced to (*R*)-2-hydroxyisocaproate by the dehydrogenase enzyme LdhA. This metabolite produces (*R*)-2-hydroxyisocaproyl-CoA via the enzyme HadA (isocaprenoyl-CoA:2-hydroxyisocaproate-CoA transferase), which renders 2-isocaprenoyl-CoA, a precursor of the synthesis of polyhydroxyalkanoates, by the enzyme constituted by HadI/HadBC (2-hydroxyisocaproyl-CoA dehydratase). This reaction is chemically difficult because the β-hydrogen that must be eliminated as a proton is not acid (pKa 4). The acidification of this hydrogen is mediated by the dehydratase HadB/HadC, ATP, and a ferredoxin-dependent activator or the archerase HadI. This is a novel mechanism to generate the necessary radicals for the dehydration, which does not depend on typical radical generators such as oxygen, coenzyme B12, nor S-adenosyl-L-methionine (Kim et al. 2005, 2006; Jain et al. 2010). The enzymes that carry out this reaction, HadB/HadC and HadI, are encoded by the genes *hadB*, *hadC*, and *hadI*, respectively (Kim et al. 2005). The 2-isocaprenoyl-CoA is reduced to isocaproyl-CoA, probably by the action of the acyl-CoA dehydrogenase enzyme encoded by the gene *acdB* and an electron transferring flavoprotein, which is encoded by the genes *etfBA*. Finally, the isocaproyl-CoA is used by HadA to produce isocaproic acid (Kim et al. 2006). The L-leucine fermentation, through the Stickland reaction, follows the stoichiometry of the Eq. 1–3 (Table 2). In the general process, 1 mol of ATP is produced from 3 mol of L-leucine. The free energy of the fermentation of 3 mol of L-leucine allows the conservation of 2 mol of ATP (≤ -70 kJ mol^{-1} ATP). This process is important in *C. difficile* since L-leucine fermentation is able to support bacterial growth (Kim et al. 2005; Jain et al. 2010); however, it can be inhibited by active surface compounds (cetultrimethylammonium bromide and desoxycholate), arsenite, iodoacetate, the redox dyes (methylviologen and phenosafranine), and metronidazole (Britz and Wilkinson 1982; Hamid et al. 1997). In the Stickland reaction, the reduction or oxidation of L-leucine can be coupled to the oxide-reduction of other amino acids. When L-leucine acts as an electron donor, L-glycine and L-proline are electron acceptors, yielding isovalerate; when L-leucine acts as electron acceptor, L-alanine or L-valine are electron donors, yielding isocaproic acid (Nisman 1954; Britz and Wilkinson 1982). In L-leucine oxidation, acetate may be an electron acceptor, producing short chain fatty acids (Eq. 4–8, Table 2) (Ato et al. 2014).

2.3 Synthesis of acetone from L-leucine metabolism

Acetone is a solvent, which can be generated in bacteria from L-leucine metabolism. This feature is widespread among the *Vibrionaceae* genus, and has been found in some strains of *Shewanellaceae* family (Nemecek-Marshall et al. 1995, 1999). Acetone formation dependent of the L-leucine pathway has been examined in the marine bacteria *Vibrio splendidus* P5 and *Photobacterium angustum* 25915 using oxygen and L-leucine as substrates. Glucose, pyruvate, and succinate act as repressors of the L-leucine-dependent acetone synthesis, suggesting that the L-leucine catabolic pathway is involved in its synthesis (Nemecek-Marshall et al. 1995, 1999). L-Leucine is catabolized by the conventional pathway up until the generation of α-ketoisocaproic acid by the activity of aminotransferases, LDH, or leucine oxidase enzymes (Stoyan et al. 1997; Beck et al. 2004; Freiding et al. 2012). The binding of oxygen atom on isopropyl moiety of keto acid seems to be the key step in acetone production, since in *Vibrio*, acetone is not obtained from acetoacetate by non-enzymatic decarboxylation (Nemecek-Marshall et al. 1999). For this purpose, α-ketoisocaproic acid catabolism may occur by two

routes; in the Route A (Fig. 1), a putative α-ketoisocaproate dioxygenase converts α-ketoisocaproic acid into 3-hydroxyisovaleric acid (3-HIV), which should be activated with CoA by an acyl-CoA synthetase under an ATP-dependent reaction generating 3HIV-CoA. Then, a putative 3-HIV-CoA lyase cleaves it, producing acetone and acetyl-CoA (Nemecek-Marshall et al. 1999). In route B (Fig. 1), α-ketoisocaproic acid follows the conventional L-leucine catabolic pathway that takes place in the *Pseudomonas* genus until the formation of 3-methylcrotonyl-CoA. Afterwards, it is diverted to acetone formation by an enoyl-CoA hydratase, which introduces a hydroxyl group on the isopropyl moiety of 3-methylcrotonyl-CoA, resulting in the formation of 3-HIV-CoA. The same route occurs in *Galactomyces reessii* to obtain 3-HIV (Dhar et al. 2002). Then, as in Route A, the cleavage of 3HIV-CoA by a putative lyase produces acetone and acetyl-CoA (Nemecek-Marshall et al. 1999). The acetone produced by these bacteria can also be used as carbon source by marine bacteria that have the ability to assimilate acetone in the environment (Nemecek-Marshall et al. 1995).

2.4 Pathways involved in flavor generation compounds from L-leucine metabolism

The α-ketoisocaproic acid, isovaleric acid, and acetyl-CoA originated during L-leucine catabolism can be diverted to the flavor-forming pathways, resulting in the synthesis of low molecular weight volatile compounds such as 3-methyl- butanal (3MBAL), 3-methyl-1-butanol (also known as isoamyl alcohol), 3-MBA, esters, and thioesters. These metabolites are related with flavor and aroma in alimentary products (Tosi and Sola 1993; van Kranenburg et al. 2002; Beck et al. 2004; Dherbecourt et al. 2008; Hazelwood et al. 2008; Liu et al. 2008; Sinz and Schwab 2012; Smid and Kleerebezem 2014). The flavor generation pathways include the Ehrlich pathway, which generates alcohols from amino acids (Hazelwood et al. 2008; Smit et al. 2009; Smid and Kleerebezem 2014). The enzymes involved in the synthesis of these volatile compounds are cytoplasmic (Smid and Kleerebezem 2014) and the activity rate-controlling step of the pathway involves the decarboxylation of α-ketoisocaproic acid (Fig. 1), since this metabolite is also a substrate of the BKD and hydroxyisocaproic dehydrogenase (HicDH) (Smit et al. 2004a, b, 2005a, b; Chambellon et al. 2009; de Cadiñanos et al. 2013).

The enzyme that carries out the non-oxidative decarboxylation of the α-ketoisocaproic acid in order to produce 3-MBAL is the branched-chain α-keto acid decarboxylase (KdcA) from *L. lactis* B1157 or the ketoisovaleric decarboxylase (KivD) in *L. lactis* IFPl730. The widely studied KdcA and KivD share a high identity between their amino acid sequences. Homotetramer conformation of both proteins is dependent of thiamin diphosphate and a divalent cation. Both enzymes carry out the breakdown of carbon–carbon bonds that are adjacent to carbonyl groups and exhibit activity over a wide substrate spectrum (De La Plaza et al. 2004; 2005a, b; Yep et al. 2006; Andrews and McLeish 2012; Li et al. 2012; Rabinovitch-Deere et al. 2013; Wei et al. 2013). Interestingly, this type of enzymes have been found in a limited number of LAB, restricting flavor-compound synthesis (Smit et al. 2004a, b, 2005a, b; Liu et al. 2008; Ziadi et al. 2010). Indirectly, 3-MBAL is also produced through BKD, which renders isovaleryl-CoA. Two additional enzymatic steps are required for 3-MBAL production (Fig. 1) (Smit et al. 2004a, b, 2009; Afzal et al. 2012). Both routes are active in *Carnobacterium maltaromaticum* LMA28 (Afzal et al. 2012).

3-MBAL is generally accumulated in low concentrations since it is subjected to further conversion into 3-methyl-1-butanol via reduction carried out by an alcohol dehydrogenase enzyme (ADH) (van Kranenburg et al. 2002; Park et al. 2009; Smit et al. 2009; Cann and Liao 2010) or into 3-MBA by oxidation by an aldehyde dehydrogenase enzyme (AldDH) (van Kranenburg et al. 2002). 3-MBA is a short chain iso-odd BCFA that can be directly produced from the conventional L-leucine catabolism as shown in Fig. 1. Transamination of L-leucine leads to the production of α-ketoisocaproic acid, which by oxidative decarboxylation is converted to isovaleryl-CoA by the BKD enzyme. Isovaleryl-CoA can be further converted into 3-MBA by the phosphotransacylase enzyme and an acyl-kinase enzyme (Kaneda 1991; Mahmud et al. 2002; Ganesan and Weimer 2004; Dherbecourt et al. 2008). In *Enterococcus faecalis* and *Lactobacillus casei*, the BKD, phosphotransacylase, and acyl-kinase are encoded in the *ptb-buk-bkdDABC* gene cluster; however, homologs of these genes in other LAB

species are located in different regions of their genomes (Liu et al. 2008). 3-MBA is produced from starter cultures in fermented meat products and during cheese ripening, contributing to the flavor of these products; however, at high concentrations, its effect is considered to be detrimental in cheese flavoring (Ganesan and Weimer 2004). 3-MBA is subject to be esterified by specific esterases, lipases, or alcohol acetyltransferases, leading to the formation of esters, such as 3-methylbutanoate ethyl ester. This compound, besides being a flavor ester, is an ethyl ester of fatty acid (FAEE), which has been proposed as biodiesel (2005a, b; Park et al. 2009; Liu et al. 2014). Volatile sulfur compounds, such as S-methyl thioesters are strong odor-producing molecules that contribute to the characteristic aroma of ripened cheeses. Although they are produced by several cheese-ripening bacteria, the synthesis of S-methyl thioesters has been reported in *Brevibacterium antiquum* and *Brevibacterium aurantiacum.* The synthesis of these compounds involves methanethiol (MTL) and acyl-CoA compounds, which can be derived from the conventional L-leucine catabolism, such as isovaleryl-CoA for producing S-methyl thioisovalerate. Other such molecules, that are precursors of S-methyl thioesters, include acetoacetate and acetyl-CoA, which produce three molecules of S-methyl thioacetate when reacting with MTL (Fig. 1). Acetoacetate also diverted to the synthesis of S-methyl thiobutyrate, since acetoacetate can be activated by an acyl-CoA synthetase or by an acetyl-CoA C-acetyltransferase with succinyl-CoA as the donor of the CoA, rendering acetoacetyl-CoA. This is reduced to produce 3-hydroxybutyryl-CoA and, by action of a hydratase, produces butyryl-CoA that, with MTL, produces S-methyl thiobutyrate. The physiological role of this kind of compounds in brevibacteria is unknown, but it is suggested that in yeast, ester synthesis could be a route to regenerate free coenzyme A without releasing high concentrations of free fatty acids with potentially cytotoxic for cells (Tosi and Sola 1993).

On the other hand, α-ketoisocaproic acid can be reduced to α-hydroxyisocaproic acid by the 2-hydroxy acid dehydrogenase enzyme (2005a, b; Chambellon et al. 2009). In *L. lactis* IFPL953, this reaction is carried out by HicDH from the family of NAD-dependent D-2-hydroxyacid dehydrogenases (Chambellon et al. 2009) or by the PanE enzyme belonging to a novel family of D-hydroxyacid dehydrogenases (Chambellon et al. 2009; de Cadiñanos et al. 2013). These enzymes catalyze the stereo-specific hydrogenation of α-keto acids, using NADH as their hydrogen donor and playing important roles in maintaining the intracellular redox balance by processing the excess from the glycolysis. However, α-hydroxyisocaproic acid is not a precursor of aroma or flavor; its production from α-ketoisocaproic acid leads to a low concentration of this precursor, negatively affecting the flux towards 3-MBAL since HicDHs compete with KdcA by the α-ketoisocaproic acid (Smit et al. 2004a, b, 2005a, b; Chambellon et al. 2009; de Cadiñanos et al. 2013). Whenever there is high reduction potential (NADH), the hydroxyacid dehydrogenase activity is dominant (Smit et al. 2004a, b), and its activity is undesirable during the fermentation of food. Thus, the selection of strains without this activity could be an interesting approach for enhancing flavor formation during fermentation of dairy products (Smit et al. 2004a, b; Wada et al. 2008; Chambellon et al. 2009). These enzymes have biotechnological interest in other fields because they are involved in catalysis of optically-active 2-hydroxyacids that are valuable for the synthesis of the semi-synthetic antibiotics and pharmaceuticals compounds (Wada et al. 2008; Chambellon et al. 2009). Interestingly, in silico predictions show that α-hydroxyisocaproic acid can be transformed into 3-MBA by the lactate 2-monoxydase enzyme, present in some LAB isolates (Liu et al. 2014).

Another strong flavor compound is 2-methylpropanal, which is commonly associated with L-valine catabolism, although this aldehyde can be originated by a chemical reaction from L-leucine, as was mentioned above, during the elaboration of cheese. In meat models, however, the chemical oxidation of α-ketoisocaproic acid is not dominant (Smit et al. 2004a, b, 2009).

The flavor-forming pathway has been extensively studied in Firmicutes, such as *Staphylococcus saprophyticus*, *S. xylosus*, *Staphylococcus carnosus*, including LAB such as *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactocillus plantarum*, among others (Ziadi et al. 2010, Gutsche et al. 2012, Sinz and Schwab 2012; Smid and Kleerebezem 2014). Many species of LAB are abundant members of the microbiota that inhabit naturally fermented food products and can be used as primary or secondary starter cultures for food fermentation; however, some have been associated

with food spoilage (Thierry et al. 2002; Dherbecourt et al. 2008; Ziadi et al. 2010; Freiding et al. 2011, 2012; Sinz and Schwab 2012; Smid and Kleerebezem 2014). This kind of bacteria displays phenotypic and genotypic heterogeneity (Freiding et al. 2011; Sinz and Schwab 2012). For example, strains of *L. lactis* subsp. *lactis* isolated from grass, corn, and fresh green peas, ferment a wide variety of sugars and produce more volatile compounds than their counterpart *L. lactis* subsp. *lactis* IL1403. The IL1403 strain uses lactose more efficiently when it is used as energy source due to their adaptation to nutrient-rich environment, such as milk (van Kranenburg et al. 2002; Alemayehu et al. 2014; Smid and Kleerebezem 2014). Strains of *L. sakei* present variation among their genome sizes (up to 25 %); only 40–50 % of genomic identity has been reported between *L. sakei* and *L. curvatus*, in spite of their high phenotypical relatedness (Freiding et al. 2011).

The synthesis of flavor compounds is carried out in processes such as milk (Ziadi et al. 2010), meat (Sinz and Schwab 2012), and natto fermentation (Kada et al. 2008), as well as in cheese ripening (2005a, b; Ziadi et al. 2010). This synthesis is important in the food industry because it contributes to the sensory perception of dairy and meat products (Smith et al. 2005a, b; Freiding et al. 2011).

It has been observed that the production of these flavor compounds is affected by growth conditions, growth phase (Masson et al. 1999), dissolved oxygen concentration (Afzal et al. 2013), the presence of uptake systems of L-leucine (van Kranenburg et al. 2002), BCAA starvation (García-Cayuela et al. 2012), insufficient production of α-ketoglutarate during cheese ripening, and also under limited transamination of L-leucine (Tanous et al. 2006; Ziadi et al. 2010). In addition, the lack of BcaT is one of the bottlenecks in the conversion of amino acids into flavor compounds, since the activity of aminotranferases are low in most LAB strains (Freiding et al. 2012; Gutsche et al. 2012; 2012).

2.5 Synthesis of bio-active compounds derived from L-leucine metabolism

2.5.1 Synthesis of iso-odd branched-chain fatty acids

Isovaleryl-CoA originated during L-leucine catabolism can be channeled into the synthesis of fatty acids comprising membrane lipids. Specifically, isovaleryl-CoA is the main precursor of iso-odd BCFA. These fatty acids are the major constituents in membranes of myxobacteria with approximately 75 % of all fatty acids content (Kaneda 1991; Mahmud et al. 2002; Beck et al. 2004; Bode et al. 2006). BCFAs (iso or anteiso) increase the fluidity of the membrane at low temperature, which can be considered as a mechanism of cold adaptation (Kaneda 1991; Mahmud et al. 2002). In fact, at 10 °C, *P. putida* increases the transport and assimilation of L-leucine, suggesting that L-leucine catabolism could provide more molecules of isovaleryl-CoA for the synthesis of iso-odd BCFAs (Mahmud et al. 2002; Fonseca et al. 2011). The synthesis of iso-odd BCFAs is similar to the synthesis of straight-chain fatty acids that occurs in *E. coli*. The differences lie in the substrate specificity of the enzyme acyl-CoA:ACP transacylase and in the condensing enzymes, which prefer substrates with acyl branched on the backbone for elongation instead of acetyl-group (Kaneda 1991; Mahmud et al. 2002). In diverse bacteria, the main iso-odd BCFAs are the iso-odd fatty acids: iso-C15:0 (13-methyl-tetradecanoic acid), iso-C17:0 (15-methyl-hexadecanoic acid), and iso-C19:0 (17-methyl-octadecanoic acid) (Beck et al. 2004; Dherbecourt et al. 2008). In myxobacteria, the iso-C17:0 is shortened through both α-oxidation and β-oxidation to produce iso-even BCFAs, such as iso-C16:0 and shortened class of iso-C17:0, including iso-C15:0, iso-C13:0, and iso-C11:0 fatty acids (Bode et al. 2005; Dickschat et al. 2005a, b).

2.5.2 Synthesis of isoprenoidal lipids and geosmin

Isovaleryl-CoA can be directed to the mevalonate pathway in myxobacteria, such as *Myxococcus xanthus*, *Nannocystis exedens*, *Stigmatella aurantiaca*; in actinomycetes, such as *Streptomyces coelicolor* and *Streptomyces citreus* (Dickschat et al. 2005a, b; Bode et al. 2006); and halophilic archaea, such as *Halobacterium salinarum.* In the mevalonate pathway, isovalery-CoA is converted into 3,3-dimethyl-acrylyl-CoA by the enzyme isovaleryl-CoA dehydrogenase; then, the 3,3-dimethyl-acrylyl-CoA is converted to 3-methylglutaconyl-CoA by a carboxylation. The 3-methylglutaconyl-CoA is subsequently transformed by the methylglutaconyl-CoA hydratase into 3-hydroxy-3-methylglutaryl-CoA, that by the activity of the 3-hydroxy-3-methylglutaryl-CoA reductase renders mevalonic acid (Dickschat et al. 2005a, b; Bode et al. 2006; Yamauchi 2010). The mevalonic acid is the precursor

metabolite for the syntheses of compounds such as isoprenoidal lipids (Yamauchi 2010) and geosmin (Dickschat et al. 2005a, b). In halophilic archaeas, membranes are characterized by isoprenoidal diethers lipids instead of ester-bound long-chain fatty acid lipids, such as those occuring in their lipid core, which contain C_{20}–C_{20}, C_{20}–C_{25}, and C_{25}–C_{25} isoprenoidal diethers. Their synthesis involves the mevalonic acid pathway, which after several phosphorylation and decarboxylation steps, leads to isopentenyl pyrophosphate (IPP) which, using a type II isomerase, produces dimethylallyl pyrophosphate (DMAPP). These intermediary compounds are considered to be universal building blocks in terpene biosynthesis. Units of IPP and DMAPP conform the isoprenoidal lipids (Dickschat et al. 2005a, b; Yamauchi 2010).

In addition, isoprenoidal lipids are involved in the synthesis of geosmin, an important odor component produced by actinomycetes, myxobacteria, and cyanobacteria bearing a strong earthy smell that has been implicated in anadromous migration of the glass eels *Anguilla anguilla*; therefore considered a signal molecule (Tosi and Sola 1993; Nawrath et al. 2008). The geosmin synthesis in streptomycetes and myxobacteria involves two units of IPP and one of DMAPP, rendering farnesyl pyrophosphate (FPP), which is cyclized to hedycaryol and further isomerized to 1(10)*E*,5*E*-germacradien-11-ol; then, octalin is produced by the loss of acetone. A subsequent reduction of octalin in combination with a 1,2-hydride shift and a final attack of water produces geosmin and an isomer of octalinis as side product (Dickschat et al. 2005a, b; Nawrath et al. 2008). In *S. coelicolor,* the gene *Sco6073* is involved in the production of geosmin and encodes for a protein with two sesquiterpene synthase domains though only one is required for geosmin biosynthesis (Gust et al. 2003).

2.5.3 *Synthesis of antimicrobials*

2.5.3.1 Synthesis of myxothiazol Myxothiazol is a natural product isolated from *Myxococcus fulvus* that is active against numerous fungi and some Gram-positive bacteria. In addition, it inhibits the electron transport via the bc_1-complex of the respiratory chain (Gerth et al. 1980; Trowitzsch et al. 1980). Myxothiazol contains a bis-thiazole moiety and a terminal amide functional group. Feeding experiments with *Stigmatella aurantiaca* showed that isovaleryl-CoA, derived from either L-leucine degradation or hydroxymethylglutaryl-CoA, conduces the generation of precursors of the thiazole moiety (Mahmud et al. 2002).

The myxothiazol biosynthetic gene cluster was identified in the genome of *S. aurantiaca* DW4/3-1, located in a coding region spanning about 60 kbp, and comprising seven genes (*mtaABCDEFG*) which encode for a megasynthetase; it was one of the first reported assembly module lines containing polyketide synthases (PKS; five modules) and nonribosomal peptide synthetases (NRPS; three modules) in a single multi-enzyme. The myxobacterial megasynthetase utilizes the isovaleryl-CoA derived from L-leucine as started unit (Silakowski et al. 1999; Moore and Hertweck 2002; Perlova et al. 2006; Wenzel and Müller 2007). The biosynthesis of myxothiazol follows a multi-step process, where 3-methylbutyrate, three molecules of acetate, two molecules of propionate, and two cysteines are condensed, giving rise to the carbon framework of the myxothiazol molecule (Perlova et al. 2006). The megasynthetase biosynthetic activity is dependent on the 4′-phosphopantetheinyl transferase (MtaA), which is also required for the formation of other secondary metabolites in *S. aurantiaca* DW4/3-1 (Gaitatzis et al. 2001). The overproduction of myxothiazol has been studied using a transposon-based strategy in *Angiococcus disciformis* d48, where it was increased in as much as 30-fold in six mutant strains. Some of the inactivated genes encode negative regulators of secondary metabolite biosynthesis. These data demonstrate that genetic manipulation of regulatory mechanisms in myxothiazol biosynthesis is a plausible tool for overproduction of this natural product (Sandmann et al. 2008; Wenzel and Müller 2009).

2.5.3.2 Synthesis of aurafurons *Stigmatella aurantiaca* and *Archangium gephyra* produce the polyketides aurafurons A and B, considered as antibiotics that inhibit the growth of some filamentous fungi. They displayed cytotoxic properties, and aurafuron B showed weak inhibition against some Gram-positive bacteria (Kunze et al. 2005; Hartmann and Kalesse 2012). The core biosynthetic gene cluster for aurafurons A and B was characterized in *S. aurantiaca* DW4/3-1. The gene core is 52.4 kbp in size and comprises 10 ORFs in a three operon arrangement, six PKS encoded in genes

aufCDEFGI, three cytochrome P_{450} monooxygenases encoded by the genes *aufABH*, and a FAD-dependent monooxygenase encoded by the gene *aufJ*. The six PKS incorporate a combined module for loading, the first extension step, and seven further modules for the incorporation of extension units. Growth assays with labeled precursors showed that isovalerate, derived from L-leucine catabolism, is the starter unit for aurafuron biosynthesis (Frank et al. 2007).

A phylogenetic analysis of the ketosynthase (KS) domains in PKS from myxobacteria and streptomycetes showed that the aurafuron biosynthetic pathway might have evolved from an earlier biosynthetic pathway. Mutations may have conferred substrate specificity and led to the loss of function of some of their domains and modules, rather than acquiring PKS encoding genes through horizontal gene transfer (Frank et al. 2007).

2.5.3.3 Synthesis of sulfangolids and kulkenon Sulfangolids and kulkenon, a structurally related compound, are sulfate esters containing secondary metabolites in different *Sorangium cellulosum* strains (Zander et al. 2012). Sulfangolid C and kulkenon bear biotechnological interest because of their anti-HIV activity (Martinez et al. 2013). In experiments using radiolabeled substrates, the starter unit of sulfangolids biosynthesis was identified as isovaleryl-CoA, derived from L-leucine, which is elongated by two extensions from methylmalonyl-CoA precursors. The final product is presumably released from the PKS assembly line (Zander et al. 2012). Sulfangolids and kulkenon share a common structural core, suggesting that kulkenon may also derive from L-leucine catabolism, although its biosynthesis has not been elucidated (Symkenberg and Kalesse 2014).

2.5.3.4 Synthesis of manumycins Manumycins are secondary polyketides of the antibiotic group, all of which are from microbial origin (Sattler et al. 1998). The first reported member, manumycin A (manumycin) was found in *Streptomyces parvulus* (Zeeck et al. 1987). Manumycins possesses interesting biological activities; for example, they show antibiotic properties against bacteria and insects, they act as potent and selective inhibitors against farnesyltransferase enzyme, and they present anti-tumor activity (Di Paolo et al. 2000; Yeung et al. 2000). L-Leucine, norleucine, and L-valine are the precursors for asukamycins of the manumycin polyketides (Moore and Hertweck 2002; Pospíšil et al. 2011).

2.5.3.5 Synthesis of aurachins Aurachins are quinoline-alkaloids with an isoprenoid (farnesyl) side chain in either the 3rd or 4th position of the quinoline nucleus. These where first isolated in *S. aurantiaca* a15 (Kunze et al. 1987). Ever since, aurachins have been identified in several *Stigmatella* strains and *Rhodococcus* sp. (Wenzel and Müller 2007). The compound family acts as strong inhibitors of the electron transport in the respiratory chain. Two members of the 4-hydroxy-2-alkylquinoline family function as signaling molecules in cell–cell communication in *P. aeruginosa* (Sandmann et al. 2007). In addition, as most of the myxobacterial antibiotics, the aurachins are active against numerous Gram-positive bacteria and have shown antimalarial activity (Schäberle et al. 2014). Bacterial growth studies reveal that aurachins are synthetized using anthranilic acid and acetate as building blocks; in addition, the farnesyl residue is constructed in parallel through any of the mevalonate and L-leucine assimilation pathways (Höfle and Kunze 2008).

The corresponding biosynthetic gene cluster has been identified in *S. aurantiaca* a15 (named *aua* core cluster). It contains seven genes that encode for a prenyltransferase (AuaA), an acyl carrier protein (ACP, AuaB), two β-ketoacyl-ACP synthase II (AuaC and AuaD), an anthranilate-CoA–ACP transferase (AuaE), an anthranilate-CoA ligase (AuaEII), and a Rieske [2Fe–2S] oxygenase (AuaF) (Sandmann et al. 2007; Pistorius et al. 2011). The AuaDCB are proteins that are representative of the type II PKS family (Sandmann et al. 2007). The AuaA enzyme catalyzes the prenylation of 2-methyl-4-hydroxyquinoline in the presence of farnesyl-diphosphate in order to produce aurachin D (Stec et al. 2011). Other genes related to aurachin biosynthesis have been found in locus II, encoding for the FAD-dependent oxido-reductase (AuaG) while locus III contains ketoreductase (AuaH), the steroid δ-isomerase (AuaI), and the FAD-dependent monooxygenase (AuaJ). The AuaG and AuaH enzymes probably catalyze the migration of the farnesyl moiety from C3 to C4 for the generation of aurachin A. Likewise, AuaI and AuaJ enzymes have been related with the generation of aurachin B (Pistorius et al. 2011; Schäberle et al. 2014).

2.5.3.6 Synthesis of ansamitocin P-4 Ansamitocins are benzenoids of ansamycin antibiotics with strong antitumor activity and are produced by actinomycetes such as *Actinosynnema pretiosum* subsp. *pretiosum*. This strain mainly produces ansamitocin P-2, bearing a propionyl moiety; ansamitocin P-3, containing a isobutyryl moiety; and ansamitocin P-4, having a isovaleryl moiety (Hatano et al. 1984). The ansamitocin's biosynthetic pathway was first described in *A. pretiosum* ssp. *auranticum* ATCC 31565, and it is composed of enzymes encoded in two gene clusters, containing about 50 ORFs that span a 96 kbp region in the genome. Four large ORFs (*asmABCD*) encode for the loading domain and seven for the chain-extension modules of a multifunctional PKS (Yu et al. 2002). The antitumor activity of the ansamytocinoids is heavily dependent of an ester side chain at C-3 position. The *asm19* gene encodes for the macrolide 3-*O*-acyltransferase, which catalyzes the attachment of the ester chain in the ansamitocins (Moss et al. 2002). Actinomycete fermentation experiments show that ansamitocin P-4 production was stimulated by L-leucine addition, indicating that the isovaleric acid released from the acyl moiety of ansamitocin P-4 was in fact originated from the ^{14}C-labeled L-leucine (Hatano et al. 1984).

2.5.3.7 Synthesis of leupyrrins *Sorangium cellulosum* So ce690 and So ce705 strains produce secondary metabolites with antifungal activity. Among them, the most structurally remarkable family corresponds to the leupyrrins (Bode et al. 2003). Leupyrrins are generated from four building blocks: the polyketide, a nonribosomal polypeptide, a dicarboxylic acid moiety, and an isoprenoid-derived element. Classical microbial growth assays indicate that isovaleryl-CoA derivated from the L-leucine catabolism is the extender unit of the polyketide biosynthesis and an intermediate in the isoprenoid element from leupyrrins. Moreover, the dicarboxylic acid moiety is formed by the condensation of α-ketoisocaproate and acetyl-CoA derived from L-leucine (Bode et al. 2004).

The leupyrrin biosynthetic cluster has been partially identified in *S. cellulosum* Soce690. The partial gene cluster resides in a 65 kbp genomic region, and it is comprised by 27 ORFs. The polyketide formation is achieved by the condensation of the unusual extender unit, 2-carboxy-3-hydroxy-5-methylhexanoyl-CoA, derived from isovaleryl-CoA. The cluster lacks of a PKS with load-modules, which would typically provide the isovaleryl-CoA to the KS domain; one possible source of the extender units is a putative aldolase (encoded in *Leu19* or *Leu20*) that catalyzes condensation between malonyl-CoA and isovaleryl-CoA to generate a 3-keto-5-methylhexanoyl-CoA intermediary to produce the corresponding dicarboxylic acid metabolite. The link to the nonribosomal polypeptides-polyketide synthase-isoprenoid on the CoA-activated end of the dicarboxylic acid may be accomplished by the putative acyl transferase Leu3, finally rendering the leupyrrins (Kopp et al. 2011).

2.5.3.8 Synthesis of carbomycin and kitasamycin Macrolides describes a group of natural antibiotics, all of which have a medium-sized lactone ring. Carbomycins and kitasamycins (also known as magnamycin and leucomycin, respectively) belong to a 16-member macrolide antibiotics family and closely related-structure natural products (Vézina et al. 1979; Ashy et al. 1980). Carbomycin is produced by *Streptomyces halstedii*, and causes inhibition of the growth of Gram-positive bacteria and mycoplasma (Ziegler and Gilligan 1981). Kitasamycins were isolated from *Streptomyces kitasatoensis*, comprising a group of 14 antibiotics which differ from the acyl substituent in position 4′ (Vézina et al. 1979); this antibiotic group has been used in the veterinary industry (Jordan and Knight 1984; Hassan et al. 1990). A radioactively labeled substrate demonstrated that the isovaleryl side chain of carbomycin is imported from L-leucine catabolism, as occurs with kitasamycin (Grisebach and Achenbach 1963a, b; Vézina et al. 1979; Li et al. 2009).

3 Bioengineering of metabolic pathways in the L-leucine metabolism

Fuels and commodity chemicals obtained from fossil fuels are essential in modern society since they are used in transportation and industry. They are critical for the synthesis of plastics, rubbers, and pharmaceutical compounds, thus indispensable for maintaining current standard of living (Cann and Liao 2008; Yan and Liao 2009). However, fossil fuels are nonrenewable resources and have contributed towards the accumulation of greenhouse gases and global warming (Foo and Leong 2013; Rabinovitch-Deere

et al. 2013). The search of biofuels and commodity chemicals derived from renewable resources and environmentally-friendly approaches respond to the rising demand for the development of sustainable technologies that may replace petroleum-based fuels. The metabolic engineering approach and Synthetic Biology have been used as platforms for biosynthesis of such compounds, using safe organisms along with a complete set of genetic tools and well-studied metabolic and regulatory networks (Connor and Liao 2009; Yan and Liao 2009; Connor et al. 2010; Rodriguez and Atsumi 2012; Foo and Leong 2013; Varman et al. 2013). The L-leucine catabolism provides some metabolites and enzymes that can be used for this goal. As presented below, intermediates of the L-leucine catabolism have the potential to be used as a raw material in synthesis of bioplastics, biofuels, and antimicrobials.

3.1 Synthesis of polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters produced by widespread microbial species as a storage material that can be used as energy and carbon source under starvation conditions. These biopolymers, considered as potential bio-plastics, are of great interest due to their biodegradable nature, which may help reduce environmental problems (Puchalka et al. 2008; Lau et al. 2011; Saika et al. 2014). The physical properties of PHAs depend on the number of carbons and side chains present in polymers (Puchalka et al. 2008). The most common PHA is poly[(*R*)-3-hydroxybutyrate] (P3HB), but is both fragile and rigid; therefore, more flexible 3-HB-based copolymers such as P(3HB-*co*-3-hydroxyvalerate) and P(3HB-*co*-3-hydroxyisocaproate) [P(3HB-*co*-3HIC)], are targets for applied research in the industry. Heterologous expression of the PHA synthase gene in *Cupriavidus necator* and *Burkholderia* sp. USM have led to the synthesis of P(3HB-*co*-3HIC) respectively using L-leucine and isocaproic acid (4-methylvaleric acid) as precursors (Lau et al. 2011; Saika et al. 2014). In order to improve the production of P(3HB-*co*-3HIC), another proposal makes use of the intermediate 2-isocaprenoyl-CoA, originating from the reductive route branch of L-leucine fermentation, which can be deviated towards synthesizing P(3HB-*co*-3HIC), as occurs using a bioengineered pathway in *E. coli* (Fig. 1). This pathway was built by recruiting the enzymes LdhA and HadAIBC from *C. difficile* and PhaCJ and PhaAB from *Aeromonas caviae* and *Ralstonia eutropha*, respectively. Once the α-ketoisocaproic acid is transformed by the enzymes LdhA and HadAIBC into 2-isocaprenoyl-CoA, which is the substrate of PhaC enzyme that produces 3-hydroxyisocaproyl-CoA. This is then fused to 3-hydroxybutyryl-CoA, rendering P(3HB-*co*-3HIC) (Fig. 1) (Saika et al. 2014). Similar metabolic network reconstruction strategies had been described in *P. putida* for engineering and designing PHAs synthesis, where L-leucine is utilized to produce the main precursor PHAs-acetyl-CoA for the synthesis (Puchalka et al. 2008).

3.2 Synthesis of biofuels

Higher chain alcohols such as 3-methyl-1-butanol are considered as a promising example of next-generation biofuels. This compound is produced through by the Ehrlich pathway, where the α-ketoisocaproic acid undergoes an irreversible decarboxylation catalyzed by KdcA or KivD, rendering an aldehyde with the release of CO_2. The aldehyde is finally reduced to 3-methyl-1-butanol by a an alcohol dehydrogenase (Connor and Liao 2009; Yan and Liao 2009; Cann and Liao 2010; Rabinovitch-Deere et al. 2013; Tashiro et al. 2015). This alcohol can also be produced from the synthesis of L-leucine, since α-ketoisocaproic acid is common in both metabolic pathways (Yan and Liao 2009; Cann and Liao 2010; Rabinovitch-Deere et al. 2013). Since α-ketoisocaproic acid is naturally generated by bacterial amino acid metabolism, the production of 3-methyl-1-butanol only requires to undergo the enzymatic steps of decarboxylation and reduction from the Ehrlich pathway. Heterologous Ehrlich pathway has been constructed in *E. coli* by introducing the *kdcA* gene (encodes to KdcA enzyme) from *L. lactis* B1157 and ADH2 from *S. cerevisiae*, leading to a more efficient processes (Andrews and McLeish 2012; Rabinovitch-Deere et al. 2013). Additionally, the 3-methyl-1-butanol esterification with acetyl-CoA via esterases from *S. cerevisiae* in a bioengineered pathway have been designed for the production of isoamyl-acetate, an ester which may be used as biodiesel (Cann and Liao 2010; Tashiro et al. 2015).

Biosynthesis of esters derived from 3-methyl-1-butanol with fatty acyl-CoA has also been achieved in

E. coli by the introduction of the genes coding for ADH2 and ARO10 from *S. cerevisiae*, an alcohol dehydrogenase and a broad-substrate-specificity 2-oxo acid decarboxylase (KdcA), respectively; and also introducing the WS/DGAT gene from *Acinetobacter baylyi* ADP1, which encodes for the diacylglycerol acyltransferase encoding genes from *Acinetobacter baylyi* ADP1. The resulting engineered *E. coli* strain was capable of synthesizing fatty acids short-chain esters (FASES) from 3-methyl-1-butanol and fatty acyl-CoA, obtaining isoamyl myristate, isoamyl palmitate, isoamyl oleate, isoamyl dodecanoate, and isoamyl 9-tetradecenoate, which are also constituent of biodiesel (Guo et al. 2014).

3.3 Genetically engineering isovaleryl attach to macrolides

Natural products cover a large portion of the drug market, particularly in the treatment of bacterial infections; however, their role in pharmaceutical research has decreased over the past two decades. In part, this is due to their often limited accessibility, as well as their structural complexity. In addition, drug-resistant bacteria are a real ever increasing problem. An approach to face this situation involves biotechnologically modifying natural product generation. Macrolides are a highly diverse group of polyketides that are used in therapeutics such as antibiotics, immune-suppressants, anti-parasites, antifungal, and antitumoral agents. All macrolides have a similar mode of action, inhibiting protein synthesis by binding to the 50S ribosome subunit (Rubinstein and Keller 1998). *Streptomycin ambofaciens* produces the antibiotic spiramycin, a 16-membered lactone ring containing three amino-sugars which are derived from L-valine catabolism. It has been designed for treatment of erythromycin-resistant infections (Park et al. 2010). Tylosin is an antibiotic produced by *Streptomyces fradiae* consisting of a polyketide lactone substituted with three deoxyhexose sugars (Bate et al. 1999). It is used in veterinary medicine (Cooper et al. 1993). Recombinant DNA technology has been applied to streptomycetes in order to produce novel macrolide antibiotics. One of these studies describes the expression of the isovaleryl transferase gene (*carE*) from *Streptomyces thermotolerans* into the producer spiramycin *S. ambofaciens;* the isovaleryl transferase enzyme mediates the addition of an isovaleryl side chain on the spiramycin molecule, producing its bioderivative: isovaleryl-spiramycin (Epp et al. 1989; Strohl et al. 1991). Isovaleryl-spiramycin (also known as bitespiramycin, biotechmycin, and shengjimycin) is a group of 4́-acylated spiramycins with isovaleryl-spiramycin I, II, and III as major components. Isovaleryl-spiramycin is also produced by a recombinant strain of *Streptomyces spiramyceticus* WSJ-1 that harbors a 4′-*O*-acyltransferase gene (*ist*) from *Streptomyces mycarofaciens* (Shang et al. 1998; Guangdong et al. 2001). Recent studies have showed that an unmodified strain of *Bacillus sp*. fmbJ can produce isovaleryl-spiramycin III (Deng et al. 2014). Similarly, a recombinant *S. fradiae* strain transformed with the *acyA*, *acyB1* and *acyB2* genes from *S. thermotolerans* was capable of producing 3-*O*-acetyl-tylosin, 3-*O*-acetyl-4′-*O*-acyltylosin, and 3-*O*-acetyl-4′-*O*-isovaleryl-tylosin. These antibiotics are widely used in veterinary treatments due to their growth inhibition effect on tylosin-resistant bacteria such as *Staphylococcus aureus* (Dıez et al. 1997). In addition, L-leucine feeding increased the relative content of isovaleryl-spiramycins produced by *S. spiramyceticus* (Li et al. 2009).

4 Conclusions

L-leucine is an amino acid that is available in almost any environment and therefore may be considered an alternative carbon and nitrogen source for bacteria growth. However, the metabolic pathways that are involved in its uptake are highly regulated. In fact, L-leucine is not a preferential substrate for bacteria; in several environmental conditions proteins involved in the uptake of L-leucine are upregulated, but not those related to the L-leucine catabolism. This fact supports the hypothesis of L-leucine being a reporter molecule for assessing amino acid availability in the environment. The main role of L-leucine is probably as a construction block in protein synthesis rather than a primary source of carbon and nitrogen. However, when bacteria exhibit an increased protein turnover due to the low-nutrient condition or prolonged starvation, L-leucine can be efficiently assimilated. Interestingly, although L-Leucine is not a preferential substrate, its catabolic pathway has been demonstrated to be dynamic and flexible, representing an example of plasticity of the bacterial metabolism, since receives,

incorporates, and redirects intermediates from catabolic pathways to other pathways for producing new compounds during the normal cell metabolism or during the degradation of synthetic compounds. On the other hand, α-ketoisocaproic acid and isovaleryl-CoA are two intermediates of the L-leucine catabolic pathway that can be channeled towards alternative pathways generating diverse compounds; some of these have a known physiological role, like iso-odd BCFAs derived from isovaleryl-CoA, which are related to cold adaptation. In addition and more importantly, compounds derived from α-ketoisocaproic acid metabolism could render new signaling molecules involved in bacteria switching from sugar to amino acid assimilation; however, further research should be carried out for supporting this finding. Such compounds display biotechnological interest, due to their roles as flavor compounds, bioplastics, biofuels, and antimicrobials; therefore, they have potential applications in different industries. Research in the field of energy generation suggests that in future decades, when oil reserves are exhausted, α-ketoisocaproic acid and isovaleric acid may be considered as important raw materials for synthesizing diverse compounds. Therefore, research of the L-leucine metabolism opens up the possibility of studying enzymes that could be target of genetic manipulation to improve the synthesis of relevant products, and even new heterologous pathways that may be built for optimizing the product yield or generating new compounds from the pathway.

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