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Review

Esters and their biosynthesis in fermented dairy products: a review

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Abstract

Esters of short-chain fatty acids are aroma-impact compounds found in fermented dairy products. These esters are responsible for fruity flavours that can be regarded either as a defect or as an attribute by the consumer. An understanding of the mechanisms of ester biosynthesis will enable control of the development of fruity flavours in fermented dairy products. The biosynthesis of flavour-active esters in dairy systems proceeds through two enzymatic mechanisms—esterification and alcoholysis. Esterification is the formation of esters from alcohols and carboxylic acids, whereas alcoholysis is the production of esters from alcohols and acylglycerols or from alcohols and fatty acyl-CoAs derived from metabolism of fatty acids, amino acids and/or carbohydrates. Alcoholysis is essentially a transferase reaction in which fatty acyl groups from acylglycerols and acyl-CoA derivatives are directly transferred to alcohols and is the major mechanism of ester biosynthesis by dairy lactic acid bacteria and yeasts.

Keywords: Esters; Flavour; Dairy microorganisms; Fermented dairy products

Contents

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1. Introduction

A range of compounds constitutes the flavour (taste and aroma) of dairy products, including volatile and non-volatile compounds. Whereas the non-volatile compounds contribute to the taste, it is the volatile compounds that contribute to both the taste and aroma of dairy products. Detailed information on the flavour of dairy products can be found in several reviews on this topic (Fox & Wallace, 1997; McSweeney, 1997; Nursten, 1997; Urbach, 1997; McSweeney & Sousa, 2000; Marilley & Casey, 2004). The aroma of dairy products is comprised of a vast array of volatile compounds, such as alcohols, aldehydes, esters, dicarbonyls, short to medium-chain free fatty acids, methyl ketones, lactones, phenolic compounds and sulphur compounds (Urbach, 1993, 1995, 1997).

Esters are common volatile constituents of some dairy products such as cheese. The contribution of esters to the flavour of dairy products is concentration-dependent. At low concentrations, esters contribute positively to the overall flavour balance; at high concentrations, they may cause a fruity flavour defect. For example, excessive levels of ethyl esters of short-chain fatty acids (typically ethyl butanoate and ethyl hexanoate) cause a fruity flavour defect in some raw and pasteurised milks, and Cheddar cheese (Bills, Morgan, Libbey, & Day, 1965; Sandine, Daly, Elliker, & Vedamuthu, 1972; Horwood, Stark, & Hull, 1987; Whitfield, 1998). Ethyl esters of the longer-chain fatty acids (C12 or above) at excessive levels may impart an undesirable soapy, tallowy odour to the product (Moio & Addeo, 1998; Moio, Dekimpe, Etievant, & Addeo, 1993a).

Various terms are used in the literature to describe the term "fruity", such as "apple-like, banana-like, pear-like, pineapple-like, strawberry-like, ester-like, ethereal, fermented and yeasty". A fruity flavour note can be a desirable attribute sought after by some consumers (Urbach, 1995, 1997). Indeed, esters are important contributors to the flavour of soft cheeses, Italian- and Swiss-type cheeses (for details, see Section 2).

Esters may also mask or attenuate the impact of off-flavours and "unclean" flavours (e.g., pungent, sharp, cowy and barney) imparted by excessive levels of certain volatile compounds such as short-chain fatty acids and phenolic compounds. Indeed, most fatty acids have considerably higher flavour perception thresholds (at ppm levels), imparting rancid, cheesy, pungent, goaty, soapy or waxy flavour notes (Brennand, Ha, & Lindsay, 1989; Collins, McSweeney, & Wilkinson, 2003). In contrast, the flavour perception thresholds of most esters are markedly lower (at ppb levels) and synergistic flavour interactions exist between different esters and between esters and other volatile components even at sub-threshold concentrations (Reddy, Lindsay, & Bills, 1969a). Therefore, esters can directly or indirectly affect flavour at levels below their individual threshold concentrations.

Cristiani and Monnet (2001) published an overview of ester synthesis by food microorganisms that are involved in dairy, meat and alcoholic beverage fermentations. Since the publication of this article, new information has emerged on the biosynthesis of esters by lipase and lactic acid bacteria by way of a transferase reaction (alcoholysis) in fermented dairy products (see Sections 4.1 and 5.3).

The aim of this article is to give an in-depth review of esters and their biosynthesis in fermented dairy products by dairy microorganisms with a view to defining the current state of knowledge of the development of fruity flavours in these products. The emphasis of this review is on the biosynthesis of esters in cheese, although ester formation in other dairy products is also touched upon briefly. Furthermore, this review is focused on the biosynthesis of fatty acid ethyl esters formed from ethanol (the most common alcohol found in cheese) that impart fruity flavours and on the biosynthesis of 2-phenylethyl esters formed from 2-phenylethanol that produce floral/rose-like flavours. In addition, the formation of *S*-methylthioesters that impart different flavour notes, (e.g., cheesy, cooked cauliflower) (Cuer et al., 1979c; Berger et al., 1999b; Khan et al., 1999) is also briefly assessed in the broad context of ester biosynthesis.

2. Esters in milk and cheese

2.1. Esters in milk

Various esters, particularly ethyl esters of fatty acids of C4–C10, are sometimes found in raw milk from cows, sheep, goats and water buffalo (Moio et al., 1993a). These esters are also found occasionally in pasteurised milk as a result of post-pasteurisation microbial contamination and microbial activity (Wellnitz-Ruen, Reineccius, & Thomas, 1982; Whitfield, Jensen, & Shaw, 2000). The esters are odour-active compounds that cause fruity off-flavours in such milk (Friedrich & Acree, 1998; Moio, Langlois, Etievant, & Addeo, 1993c). The biological origin of these esters in milk (raw and pasteurised) and the microorganisms responsible are discussed in Section 5.4. The type, aroma profile, flavour thresholds and quantities of the esters frequently found in milk are presented in Table 1.

2.2. Esters in cheese

Esters are common volatile components of cheese. A range of esters is found in the major cheese types. Of the esters identified in a variety of cheese, the five ethyl esters of the straight-chain fatty acids of C2–C10 are

Table 1
Aroma, thresholds and quantities of esters frequently found in milk and cheese[a]

Ester	Aroma	Threshold (ppm)[b]	Quantity (ppm)[b]	Milk/Cheese
Ethyl Acetate	Solvent	5 (water/milk)	52–99	Ewe cheese
	Fruity	22 (oil/butter)	0.05–0.25	Parmigiano-Reggiano
	Pineapple			
Ethyl Butanoate	Apple	0.005 (milk)	0.026–0.152	Fruity milk
	Banana	0.0035–0.028 (oil)	0.146	Goat cheese
	Sweet	0.6 (oil/butter)	0.28–1.82	Fruity Cheddar
	Fruity	0.00013–0.45 (water)	0.026–0.05	Emmental
	Fragrant		0.07	Mozzarella
			0.124–0.362	Grana Padano
			0.25–1.14	Parmigiano-Reggiano
Ethyl Hexanoate	Banana	0.005 (milk)	0.20–0.268	Fruity milk
	Pineapple	0.2	9–14	Ewe cheese
	Sweet	0.02–0.04 (oil)	0.258	Goat cheese
	Fruity		0.43–2.08	Fruity Cheddar
	Wine-like	0.001 (water)	0.062–0.142	Emmental
	Brandy	0.9 (oil/butter)	0.05	Mozzarella
	Powerful		0.03–0.08	Gorgonzola
			1.23	Danish Blue
			0.215–0.993	Grana Padano
			2.58	Parmigiano- Reggiano
Ethyl Octanoate	Pear	0.9	0.9–5	Ewe cheese
	Sweet		0.48	Goat cheese
	Fruity	0.0001 (water)	0.28–1.07	Fruity Cheddar
	Banana		0.06	Mozzarella
	Pineapple		0.1–0.13	Gorgonzola
	Apricot		0.037–0.229	Grana Padano
	Wine		0.25–1.62	Parmigiano-Reggiano
	Floral			
Ethyl Decanoate	Apple	1.5	1.4–5	Ewe cheese
	Brandy		0.901	Goat cheese
	Grape-like	0.2 (10% ethanol)	0.002–0.02	Gorgonzola
	Fruity		0.05	Mozzarella
	Oily		0.002–0.03	Grana Padano
			0.25–1.0	Parmigiano-Reggiano
			11.48	Danish Blue
2-Phenylethyl acetate	Floral	0.137 (oil or butter)	0.083	Goat cheese
	Rose-like	0.02 (water)	0.02	Mozzarella
	Honey		0.0005–1.0	Camembert

[a] Data are taken from McGugan et al. (1975), Wellnitz-Ruen et al. (1982), Meinhart and Schreier (1986), Roger et al. (1988), Moio et al. (1993b), Preininger and Grosch (1994), Molimard and Spinnler (1996), Le Quere et al. (1998), Moio and Addeo (1998), Sablé and Cottenceau (1999), Dahl et al. (2000), Moio et al. (2000), Alewijn et al. (2003) and Qian and Reineccius (2003c).
[b] $mg\,L^{-1}$ or $mg\,kg^{-1}$ cheese. Unless specified otherwise, the medium in which threshold values were obtained is not known.

most frequently found (Table 1). There are also numerous other esters that are less frequently found (Table 2). Although these esters are normally present at low levels, they may still contribute to the overall flavour balance of cheese because of synergistic flavour interactions between different esters and between esters and other volatile components at concentrations below their flavour thresholds (Reddy et al., 1969a).

The type and concentration of esters found in cheeses vary between cheese varieties and with manufacturing conditions. Likewise, whether the flavour impact from esters is discernible or not is also dependent on the cheese type, ester type and ester concentration. For example, a range of esters are frequently found and identified as odourants in some cheese types (e.g., Cheddar and Dutch-type) that are not normally perceived to be fruity (see below). In these cheese types, the esters probably contribute to the overall flavour complexity without a fruity intensity that is considered a defect. Although esters have been identified as odorants

Table 2
Aroma, thresholds and quantities of esters less frequently found in milk and cheese[a]

Ester	Aroma	Threshold (ppm)[b]	Quantity (ppm)[b]	Milk/cheese
Ethyl formate	Sharp			Ewe cheese
Methyl acetate	Ethereal			Dutch cheese
	Sweet			Cheddar
				Parmesan
				Gruyére
Propyl acetate	Pineapple	30	1.2–41	Ewe cheese
Pentyl acetate	Fruity	0.04 (water)		Ewe cheese
Hexyl acetate	Fruity/pear	3.5	3.1–4.1	Ewe cheese
Ethyl lactate	Fruity			Ewe cheese
Ethyl propionate	Fruity/rum	0.005 (water)	0.03	Parmigiano-Reggiano
1-Methylpropyl propionate				Swiss
Butyl propionate	Ethereal			Swiss
Methyl butanoate	Fruity	0.04 (water)	Trace	Ewe cheese
		4.7 ppb (air)		Parmesan
				Grana Padano
Propyl butanoate	Sharp			Swiss
	Pungent		Trace	Grana Padano
Ethyl isobutanoate	Green	0.0008 (oil)	0.01	Mozzarella
Ethyl valerate	Fruity	0.0015 (water)	12–16	Ewe cheese
Ethyl isovalerate	Fruity	0.0002 (water)	6.9–10	Ewe cheese
			0.01	Mozzarella
Methyl hexanoate	Pineapple	0.13 (water)		Ewe cheese
Ethyl heptanoate	Fruity	0.002 (water)		Raw milk
	Wine-like		0.007	Goat cheese
	Brandy			Ewe cheese
			Trace	Grana Padano
Ethyl nonanoate	Fruity			Raw milk
			0.008	Goat cheese
			Trace	Grana Padano
Methyl decanoate	Fruity			Ewe cheese
Ethyl 9-decenoate			0.034	Goat cheese
Ethyl undecanoate	Cognac		0.019	Goat cheese
Methyl dodecanoate	Soapy			Ewe cheese
Ethyl dodecanoate	Fruity			Raw milk
	Floral			Ewe cheese
			0.057	Goat cheese
			0.01	Mozzarella
			Trace	Grana Padano
Ethyl tetradecanoate	Soapy		4.01	Danish Blue
	Tallowy		0.038	Goat cheese
	Waxy			Ewe cheese
Ethyl hexadecanoate	Soapy	>2 (water)	1.29	Danish Blue
	Waxy			Raw milk

[a] Data are taken from Martínez-Castro et al. (1991), Moio et al. (1993a, b), Barbieri et al. (1994), Preininger and Grosch (1994), Yang and Min (1994), Molimard and Spinnler (1996), Engels et al. (1997), Le Quere et al. (1998), Moio and Addeo (1998), Sablé and Cottenceau (1999), Dahl et al. (2000), Ortigosa et al. (2001), Alewijn et al. (2003) and Qian and Reineccius (2003c).

[b] $mg\,L^{-1}$ or $mg\,kg^{-1}$ cheese. Unless specified otherwise, the medium in which threshold values were obtained is not known.

of various cheese types, there are few correlations between esters and sensory properties of cheese, except for Cheddar cheese with a fruity defect and Italian-type cheeses (see below). In the following discussion, an attempt is made to summarise the type of esters identified in major cheese varieties and to assess their potential contributions to sensory properties.

Ethyl esters of fatty acids of C2–C10 are volatile components of Cheddar and Dutch-type cheeses (e.g., Gouda and Edam) (Banks, Brechany, Christie, Hunter, & Muir, 1992; Arora, Cormier, & Lee, 1995; Christensen & Reineccius, 1995; Engels, Dekker, de Jong, Neeter, & Visser, 1997; Suriyaphan, Drake, Chen, & Cadwallader, 2001). These esters have been further identified as odourants that contribute to the aroma of Cheddar cheese using gas chromatography-olfactometry (GC-O) and dynamic headspace dilution assay (Zehentbauer & Reineccius, 2002). Nonetheless, esters are present in minute quantities only (<1 ppm) in Gouda and Cheddar cheese not normally associated with fruitiness (Alewijn, Sliwinski, & Wouters, 2003). However, excessive levels of these ethyl esters of fatty acids of C4–C10 are found in Cheddar cheese with a fruity defect (Bills et al., 1965; McGugan et al., 1975; Horwood et al., 1987).

Fruitiness is generally an attribute of Italian-type cheeses (e.g., Parmigiano Reggiano, Parmesan and Grana Padano). Ethyl esters of fatty acids of C2–C10 are the predominant volatile compounds that are responsible for the fruity character of these cheeses (Dumont, Roger, & Adda, 1974; Meinhart & Schreier, 1986; Barbieri et al., 1994; Virgili et al., 1994; Moio & Addeo, 1998; Qian & Reineccius, 2002, 2003a–c). In addition, 18 other esters are present at relatively low levels in Parmesan and Grana Padano cheese (Barbieri et al., 1994; Moio & Addeo, 1998). Using GC-O, it has now been demonstrated that these esters are primarily responsible for the characteristic fruity aroma perceived in this type of cheese (Qian & Reineccius, 2002; Boscaini, van Ruth, Biasioli, Gasperi, & Mark, 2003). In Mozzarella cheese that is not fruity, 12 esters have been identified with ethyl 3-methyl butanoate and ethyl esters of fatty acids of C4–C10 being predominant (Moio et al., 1993b).

Swiss-type cheeses (e.g. Emmental and Gruyére) contain not only ethyl esters of fatty acids of C2–C8 common to other cheese types, but also esters unique to these cheeses (e.g., ethyl propionate, 1-methyl propyl propionate, butyl propionate) (Imhof & Bosset, 1994; Preininger & Grosch, 1994; Yang & Min, 1994; Engels et al., 1997; Noël, Boyaval, Thierry, Gagnaire, & Grappin, 1999). Thierry, Maillard, and Le Quere (1999) identified considerably more low molecular weight esters in the aqueous phase (16 esters) than in the oil phase (9 esters) of a Emmental cheese or in the cheese itself (10 esters), suggesting that esters are more easily released from the aqueous phase. Most esters in the Emmental cheese increased by 4–20 fold during ripening (Thierry et al., 1999). Esters are positively associated with the sweet odour of Swiss-type cheeses (Lawlor, Delahunty, Wilkinson, & Sheehan, 2001). The unique esters in Swiss-type cheeses and their appearance in dairy fermentations where only propionibacteria are present (Liu, Holland, & Crow, unpublished data) indicate that the role of propionibacterial esterases/lipases should be investigated further (see Section 5.6).

Esters are prevalent in mould-ripened cheeses (e.g., blue cheeses) and surface mould-ripened cheeses (e.g., Camembert). Gallois and Langlois (1990) identified 57 esters in French blue cheese with the predominant esters being methyl and ethyl esters of fatty acids of C2–C10. It has now been verified that esters, together with methyl ketones and fatty acids, are the volatile flavour compounds central to the characteristic Blue cheese aroma (Qian, Nelson, & Bloomer, 2002; Alewijn et al., 2003). Indeed, the fruity/sweet odour of blue-type cheeses is positively correlated with the level of esters in these cheeses (Lawlor, Delahunty, Sheehan, & Wilkinson, 2003). In addition, 2-phenylethyl acetate is an ester frequently found in Camembert cheese (Roger, Degas, & Gripon, 1988; Kubickova & Grosch, 1997, 1998a, b). Moio, Piombino and Addeo (2000) found 17 and 21 esters in, respectively, the creamy and natural types of Gorgonzola cheese (an Italian soft blue-veined cheese) with ethyl butanoate and ethyl hexanoate being two of the most important aroma compounds. Molimard and Spinnler (1996) and Sablé and Cottenceau (1999) reviewed the volatile flavour compounds, including esters, in mould-ripened and surface mould-ripened cheeses and additional information on esters in these cheese types can be found in these two review articles.

Esters are part of the aroma array of cheeses made from goats' and ewes' milk (e.g., Manchego, Feta, Serra da Estrela and Roncal), but the number and type of esters found vary between cheese varieties. A total of 11 esters are found in a soft goat cheese, with ethyl esters of fatty acids of C4–C10 being the most abundant (Le Quere, Pierre, Riaublanc, & Demaizieres, 1998). Twelve methyl and ethyl esters of fatty acids of C4–C14 are found in Manchego cheese (Martínez-Castro, Sanz, Amigo, Ramos, & Martín-Alvarez, 1991). In contrast, only four esters of up to fatty acids of C4 are found in Feta cheese (Horwood, Lloyd, & Stark, 1981). Dahl, Tavaria and Malcata (2000) found eight esters including ethyl isovalerianate (2-methyl butanoate, a branched-chain fatty acid) in Serra da Estrela cheese with ethyl, propyl and hexyl esters of acetate being predominant. Ethyl acetate is the most abundant ester among the seven esters identified in Roncal cheese (Izco & Torre, 2000; Ortigosa, Torre, & Izco, 2001). The prevalence of acetate esters in ewes' milk cheese may be related to the unique ripening flora such as enterococci and yeasts

associated with this type of cheese, especially when raw milk is used (Dahl et al., 2000; Papademas & Robinson, 2000).

Branched-chain fatty acids, typically 4-methyloctanoic and 4-ethyloctanoic acids, are characteristic of the fatty acid composition of milkfats of goats' and ewes' milk (Ha & Lindsay, 1991, 1993; Alonso, Fontecha, Lozada, Fraga, & Juarez, 1999). Surprisingly, to the best of our knowledge, esters of these branched-chain fatty acids have not been reported in cheeses made from goats' and ewes' milk. This could be due to the generally low concentrations of these esters that are beyond the detection limits of current analytical methods (GC and GC-MS with or without solid-phase microextraction, SPME). Another reason could be the selectivity of esterases/lipases and/or the ripening flora, as reported elsewhere (Ha & Lindsay, 1993; Gaborit, Menard, & Morgan, 2001; Morgan & Gaborit, 2001). Esters of branched-chain fatty acids have low odour thresholds and may be beneficial for masking the typical goat-like and mutton-like flavours.

It should be noted that other volatile compounds such as ketones and methyl ketones also impart fruity notes to cheeses, besides esters. However, the concentrations of these compounds in most cheese varieties are generally low, compared with the concentrations of the esters. The exceptions are mould-ripened and surface mould-ripened cheeses, which contain relatively high levels of ketones and methyl ketones (Kinsella & Hwang, 1976; Gripon, 1993, 1997; Sablé & Cottenceau, 1999; Moio et al., 2000).

S-methylthioesters (e.g., *S*-methylthioacetate and *S*-methylthiopropanoate) imparting different flavour notes (cheesy, cooked cauliflower) are frequently found in mould- and surface mould-ripened cheeses (Berger et al., 1999b; Khan et al., 1999; Sablé & Cottenceau, 1999; Lecanu, Ducruet, Jouquand, Gratadoux, & Feigenbaum, 2002), in addition to the fruity esters mentioned above. *S*-methylthioacetate and *S*-methylthiopropanoate are also found in Swiss-type cheese (Yang & Min, 1994; Thierry & Maillard, 2002). *S*-methylthioesters are very potent odourants with threshold values ranging from 5 to 200 ppb (Cuer et al., 1979c). Very low levels of these thioesters are, therefore, expected to impact considerably on cheese flavour.

Clearly, esters are an integral part of the volatile make-up of cheeses and are important to the component balance of cheese flavour. Esters often contribute to the overall cheese flavour profile either in their own right or through synergistic interactions with other flavour components. Furthermore, as with beer and wine (Pretorius, 2000; Verstrepen et al., 2003a), esters probably contribute most to the 'fermentation bouquet' of cheese. More work is needed to evaluate the contribution of esters to the quality attributes of cheese varieties not normally associated with fruitiness, in addition to the more studied Italian-type cheese.

Caution should be exercised when correlating esters with the sensory attributes of cheese. This is because the concentration of esters is not always positively associated with fruitiness and can be correlated with other flavour attributes. For example, whereas esters are positively associated with the sweet odour of Swiss-type cheeses, esters are also positively correlated with acidic, peppery, salty and silage-like flavours in other cheese types (Lawlor et al., 2001). Also, the nutty flavour of Emmental cheese is found to be positively correlated with the concentration of ethyl acetate, whereas the sweet flavour of Dubliner cheese is negatively correlated with the concentration of ethyl hexanoate (Lawlor, Delahunty, Wilkinson, & Sheehan, 2002). Further, it has been found that artisanal Manchego cheese containing more esters (both type and amount) than an industrial counterpart had no fruity notes, whereas the industrially produced cheese did (Gomez-Ruiz, Ballesteros, Gonzalez Vinas, Cabezas, & Martinez-Castro, 2002).

The above examples indicate flavour component interactions such as masking of the fruity aroma of esters by the more intense odours imparted by higher levels of free fatty acid levels. As stated by Lawlor et al. (2001), 'associative' is not necessarily 'causative' and a statistical correlationship between variables (sensory, volatile and compositional) does not imply a cause-and-effect situation. Clearly, information gained from such correlation studies must be interpreted with caution.

3. Mechanisms of ester biosynthesis in fermented dairy products

The traditional view is that the biosynthesis of esters in fermented dairy products involves a two-step process. In this two-step process, glycerides present in milk fat are first hydrolysed by lipases to liberate free fatty acids and glycerol, followed by the esterification of free fatty acids with an alcohol (in most cases, ethanol) to produce esters catalysed by esterases (Morgan, 1976; Kempler, 1983; Fox & Wallace, 1997; Fox, Guinee, Cogan, & McSweeney, 2000, Chapter 12).

Lipases are generally defined as the enzymes that catalyse the hydrolysis of carboxyl ester bonds in water-insoluble glycerides in an emulsion to release fatty acids and glycerol. Esterases are defined as the enzymes that hydrolyse carboxyl ester linkages in water-soluble substrates (e.g., slightly water-soluble short-chain glycerides and aliphatic esters) in aqueous solutions. Although lipases and esterases normally catalyse the hydrolysis of glycerides and esters, these enzymes can also synthesise esters under certain conditions (see Sections 4.1 and 5.3).

Table 3
Mechanisms of ester biosynthesis in dairy and non-dairy microorganisms

Enzyme	Reaction pathway	Microorganism
Esterase/lipase	Esterification $R_1COOH + R_2OH \rightarrow R_1COOR_2 + H_2O$	Lactic acid bacteria, propionibacteria, pseudomonads, smear bacteria, yeasts, moulds
Esterase/lipase	Thioesterification $RCOOH + CH_3SH \rightarrow RCOSCH_3 + H_2O$	Lactic acid bacteria, smear bacteria,
Alcohol acyltransferase (esterase/lipase)	Alcoholysis $R_1COOR_2 + R_3OH \rightarrow R_1COOR_3 + R_2OH$	Lactic acid bacteria, moulds
Alcohol acyltransferase	Alcoholysis $R_1OH + R_2COSCoA \rightarrow R_2COOR_1 + CoASH$	Yeasts
Alcohol acetyltransferase	Alcoholysis $ROH + CH_3COSCoA \rightarrow CH_3COOR + CoASH$	Yeasts
Alcohol acetyltransferase	Transthioesterification $CH_3SH + CH_3COSCoA \rightarrow CH_3COSCH_3 + CoASH$	Yeasts
Methyl formate synthase (alcohol dehydrogenase?)	Dehydrogenation $CH_3OH + HCHO \rightarrow CH_2(OH)OCH_3 + NAD^+ \rightarrow HCOOCH_3 + NADH + H^+$	Methylotrophic yeasts (*Candida boidinii*, *Pichia methanolica*)
Hemiacetyl dehydrogenase (alcohol dehydrogenase?)	Dehydrogenation $R_1OCH(OH)R_2 + NAD^+ \rightarrow R_2COOR_1 + NADH + H^+$	*Saccharomyces cerevisiae*, *Candida utilis*

In addition to esterases and lipases, there are other enzymes that are involved in ester biosynthesis. For example, in yeasts, several enzymes are involved such as alcohol acetyltransferases, methyl formate synthase and hemiacetal dehydrogenase (see Section 5.1).

The different mechanisms of ester biosynthesis in both dairy and non-dairy microorganisms are summarised in Table 3. The following sections will discuss the current knowledge of the mechanisms operating in different biological systems.

Although the focus of this review is on the enzymatic synthesis of esters, it should be pointed out that esters, including *S*-methylthioesters, can be formed non-enzymatically (chemically). We have found that there is some spontaneous formation of esters from alcohols and artificial glycerides, though not from ethanol and milk fat (Liu, Holland, & Crow, unpublished data). There is also some spontaneous synthesis of *S*-methylthioesters from methanethiol and fatty acyl-CoA (Helinck, Spinnler, Parayre, Dame-Cahagne, & Bonnarme, 2000). The contribution to cheese flavour of spontaneous formation of esters is not expected to be as significant as the enzymatic synthesis, because the latter process gives a higher yield. Practically, both the spontaneous and enzymatic formation of esters may contribute to the development of cheese flavour, though it is difficult to quantify their relative contributions in cheese due to lack of information.

4. Ester biosynthesis by lipases and role of water activity

4.1. Ester biosynthesis by lipases

Lipases, extracted and purified to varying degrees from mammalian and fungal origins, are used in the manufacture of a range of fermented dairy products such as Italian-type cheeses and enzyme-modified cheese (Birschbach, 1992; Ha & Lindsay, 1993; Kilcawley, Wilkinson, & Fox, 1998). The primary role of lipases in these products is to promote the breakdown of triglycerides in milk fat to release free fatty acids to enhance flavour (Woo & Lindsay, 1984; O'Connor & Lai, 1996). Despite the extensive use of lipases, their role in the biosynthesis of esters has not been defined in dairy systems.

Lipases can catalyse the synthesis of esters under certain conditions. This catalysis can be by four types of reactions:

(i) esterification ($R_1COOH + R_2OH \rightarrow R_1COOR_2 + H_2O$);
(ii) alcoholysis ($R_1COOR_2 + R_3OH \rightarrow R_1COOR_3 + R_2OH$);
(iii) acidolysis ($R_1COOR_2 + R_3COOH \rightarrow R_3COOR_2 + R_1COOH$);
(iv) transesterification ($R_1COOR_2 + R_3COOR_4 \rightarrow R_1COOR_4 + R_3COOR_2$)

(Malcata, Reyes, Garcia, Hill, & Amundson, 1992). Interesterification is a general term used for alcoholysis, acidolysis and transesterification. Acidolysis and transesterification are commonly employed to structurally modify lipids to produce nutritionally functional fats and oils (Willis, Lencki, & Marangoni, 1998) and will not be discussed further in this review. The following discussion is focused on ester synthesis via esterification and alcoholysis, as these two ester-producing reactions have potential applications in dairy systems in terms of ester biosynthesis for flavour.

There is little information on the biosynthesis of esters by lipases in dairy systems such as natural cheese and enzyme-modified cheese. However, a wealth of information is available in the literature that suggests that lipases can indeed synthesise esters via esterification, albeit mostly in low-water/non-aqueous systems (e.g., organic solvents) (for more information, see a review by Yahya, Anderson, & Moo-Young, 1998). For example, mammalian and fungal lipases can catalyse the synthesis of short-chain flavour esters from alcohols and carboxylic acids in organic solvents (Langrand, Rondot, Triantaphylides, & Baratti, 1990; Dias, Vilas-Boas, Cabral, & Fonseca, 1991; Kim, Altreuter, Clark, & Dordick, 1998; Lai & O'Connor, 1999).

In contrast to the biosynthesis of esters by lipases via esterification in low-water systems mentioned above, some fungal lipases can synthesise esters by esterification in an aqueous environment (Okumura, Iwai, & Tsujisaka, 1979; Ishii et al., 1990). Perhaps most importantly, a lipase from *Candida cylindracea* can first hydrolyse milk fat, then esterify the released fatty acids with ethanol to produce ethyl esters in both aqueous and non-aqueous media (Kanisawa, 1983; Yu, Rizvi, & Zollweg, 1992). A purified lipase from *Can. deformans* can catalyse ester synthesis in aqueous media by esterification but not by alcoholysis (Boutur, Dubreucq, & Galzy, 1995). Control of water content (water activity, see Section 4.2) is important for ester synthesis by lipases via esterification. The use of lipases that are able to synthesise esters via esterification in an aqueous environment removes the need to control water activity and more importantly, the use of organic solvents can be eliminated.

Ester synthesis by lipases via alcoholysis is also known. It appears to be common in aqueous systems in which fatty acyl groups from acylglycerols are directly transferred to alcohols. Both alcoholysis and hydrolysis can occur in the presence of alcohols in an aqueous environment. The lipases thus exhibit acyltransferase activities in which the acyl acceptor may be either an alcohol (alcoholysis) or water (hydrolysis). For example, a lipase produced by *Aeromonas hydrophila* is actually an acyltransferase that preferentially catalyses acyl transfer from lipids to alcohols in an aqueous environment (Robertson, Hilton, & Buckley, 1992). Also, a lipase from *Can. parapsilosis* catalyses ester synthesis mainly by alcoholysis (Briand, Dubreucq, & Galzy, 1994, 1995; Lecointe, Dubreucq, & Galzy, 1996).

We have recently demonstrated that a commercial lipase from *Rhizomucor miehei* catalysed the synthesis of ethyl butanoate from ethanol and tributyrin (an artificial triglyceride) in an aqueous environment and in an aqueous cheese-based medium supplemented with ethanol (Liu, Holland, & Crow, 2003a). In the same study, we showed that the lipase did not catalyse the formation of ethyl butanoate from butanoic acid and ethanol in an aqueous environment. Further, the addition of butanoic acid along with ethanol to the cheese-based medium did not enhance ethyl butanoate synthesis. This study suggested that alcoholysis is the mechanism of ester synthesis catalysed by this lipase in an aqueous environment such as dairy systems.

In addition to alkyl esters, it has been demonstrated that two immobilised fungal lipases (Lipozyme^{TM} and Novozyme^{TM}) can catalyse the synthesis of thioesters from a carboxylic acid and a thiol (thioesterification) and from an ester and a thiol (transthioesterification) in low-water systems (Cavaille-Lefebvre & Combes, 1997; Caussette, Marty, & Combes, 1997). These findings suggest that lipases may play a role in the biosynthesis of flavour thioesters in dairy systems, provided that the key precursors, thiols (e.g., methanethiol) and fatty acids, are available in sufficient amounts and that the water activity is favourable (see Section 4.2).

To sum up, lipases can catalyse the synthesis of flavour esters by esterification or alcoholysis in an aqueous environment. It can be envisaged that lipases that catalyse ester synthesis by alcoholysis may also catalyse ester synthesis by esterification at least in low-water systems. However, lipases that catalyse ester formation by esterification may not necessarily catalyse ester production by alcoholysis. The mechanism of enzymatic synthesis of esters varies with lipases and should be determined experimentally for each lipase. In dairy systems such as cheese, possibly both esterification and alcoholysis contribute to ester synthesis. We reason that alcoholysis may play a greater role in ester synthesis in cheese, considering that few lipases can catalyse esterification in an aqueous environment and that the water activity of most cheeses is unfavourable for esterification (see Section 4.2).

4.2. Role of water activity in ester synthesis

Water is a product of the esterification reaction between an alcohol and a carboxylic acid. Water activity is one of the key parameters affecting lipase-catalysed reactions (hydrolysis and esterification) (Halling, 1994; van Camp & Huyghebaert, 1998; Yahya et al., 1998). A number of studies have demonstrated that water acts as a competitive inhibitor of lipase-catalysed esterification,

though lipases respond differently to water activity changes (Monot, Borzeix, Bardin, & Vandecasteele, 1991; Valivety, Halling, & Macrae, 1993; Svensson, Wehtje, Adlercreutz, & Mattiason, 1994; Wehtje & Adlercreutz, 1997a, b). In general, in the presence of a lipase, a carboxylic acid and an alcohol, a high water activity will favour hydrolysis, whereas a low water activity will favour esterification.

The water activity of dairy systems varies with products. For example, the water activity of cheese is usually in the range of 0.70–0.99, though most cheese types have water activities above 0.90 (Rüegg & Blanc, 1981; Marcos, 1993). The water activity of other fermented dairy products ranges from 0.97–0.99 (Rüegg, 1985). Therefore, the water activity of dairy systems is generally unfavourable for ester biosynthesis via esterification. However, by definition, water activity applies only to an equilibrium state in a system. Because cheese is a very dynamic system, water activity of cheese is dynamic, too. Indeed, there are considerable zonal variations in water activity within the cheese matrix (Rüegg & Blanc, 1981; Marcos, 1993) and there may well be "pockets" in cheese in which the water activity is more favourable for esterification.

Water activity can impact on microbial growth and metabolic activity, cheese composition, texture and flavour (Rüegg, 1985; Marcos, 1993; Liu, Asmundson, Gopal, Holland, & Crow, 1998a). However, the impact of water activity on lipase-catalysed reactions (hydrolysis and esterification) is dependent on the enzyme(s): some give increasing esterifying activity with increasing water activity, whereas others give decreasing esterifying activity with increasing water activity (Wehtje & Adlercreutz, 1997a, b).

Ha and Lindsay (1992) observed an increased formation of ethyl esters of fatty acids of C4–C10 but decreased volatile free fatty acid content in cheese bases (pre-lipolysed with a mammalian lipase) held at water activity of 0.90 in the presence of ethanol, compared with their formation in the absence of ethanol. This observation implies that the lipase might have catalysed the synthesis of these ethyl esters either by esterification or by alcoholysis or both in the cheese bases. Therefore, we hypothesise that the mammalian and microbial lipases can synthesise flavour esters via esterification and/or alcoholysis in dairy systems, provided that ethanol is available and/or that the water activity is suitable. This hypothesis needs to be corroborated with definitive evidence, ideally in dairy systems. Indeed, evidence is available that esterases of several lactic acid bacteria (LAB) seem to be able to synthesise ethyl esters of short-chain fatty acids in a reaction mixture primarily comprised of casein, NaCl, calcium phosphate, alcohols and fatty acids at low water activity (0.85–0.925), albeit from high substrate concentrations (1000 ppm each) (Fenster, Rankin, & Steele, 2003b).

5. Ester biosynthesis by dairy microorganisms

There are three key factors that determine ester biosynthesis by dairy micoorganisms: enzymes, substrates and environment. There is not enough published information in any one group of microorganisms to define clearly all three factors and so, applications for controlling ester production cannot be developed in a systematic manner.

The enzymes involved in ester biosynthesis in dairy systems are largely determined by the type of microorganisms present and their growth conditions, although some enzymes can be added to dairy systems as commercial preparations such as lipases/esterases. The substrates required for the production of esters vary depending on the enzymes present. For example, the fatty acid moieties of esters may originate directly or indirectly from milkfats and amino acids, from fermentation products (e.g., propioniate and acetate) and/or from fermentation intermediates (particularly CoA derivatives). Alcohols (usually ethanol) can be produced either by microorganisms with the crucial ester-synthesising enzymes such as alcohol acetyltransferases and/or esterases (e.g., yeasts) or by other microorganisms from substrates such as carbohydrates and amino acids (e.g., branched-chain alcohols are derived from corresponding amino acids). Once the crucial enzymes are present, then the ester-producing reactions are determined by the concentrations of reactants (substrates) and the environment.

In all the dairy systems studied, it is not possible to define the rate-determining steps due to lack of information. Therefore, references to microorganisms (particularly yeasts) from other sources are inevitable with regard to ester biosynthesis. We discuss below the key groups of dairy microorganisms and their potential to synthesise esters, highlighting current knowledge.

5.1. Ester biosynthesis by dairy yeasts

Yeasts are ubiquitous and are present in dairy products such as milk, yoghurt, cheese and fermented milk beverages (e.g., kefir and koumiss) (Fleet, 1990). The impact of yeasts in dairy products can be two-fold: a positive contribution to the fermentation and maturation of dairy products (e.g., aroma formation, lipolysis and proteolysis) or a negative impact causing spoilage (e.g., gas production, off-flavour formation and discolouration) (Jakobsen & Narvhus, 1996).

The positive impact of yeasts on cheese ripening is not well defined, despite the fact that yeasts are not only part of the starter cultures of surface-ripened cheeses but are also present in the inner region of both soft and hard cheeses (Callon, Chataud, Vanderbecken, & Larpent, 1994; Bockelmann, 1999, 2002; Corsetti, Rossi, & Gobbetti, 2001). Much research has been devoted to

understand the contribution of yeasts to cheese ripening with regard to lipolysis, proteolysis and glycolysis (Wyder & Puhan, 1999; van den Tempel & Jakobsen, 2000; Addis, Fleet, Cox, Kolak, & Leung, 2001; Hansen & Jakobsen, 2001; Guerzoni et al., 2001; Lucia, Daniela & Rosalba, 2001; Suzzi et al., 2001). By contrast, relatively little is known about the formation of volatile aroma compounds, including esters, by dairy yeasts.

Several studies have investigated the relationships between sensory characteristics and the production of aroma compounds in cheese curd co-cultured with surface bacteria and yeasts such as *Kluyveromyces lactis*, *Debaryomyces hansenii* and *Yarrowia lipolytica* (Martin et al., 1999; Martin, Berger, Le Du, & Spinnler, 2001; Martin, Berger, & Spinnler, 2002). It was found that the yeasts (especially *Klu. lactis*) were able to produce a range of volatile compounds (alcohols, aldehydes, esters and terpenes) when cultured alone or in association with bacteria. It was the esters (ethyl acetate and ethyl butanoate) that were largely responsible for the fruity aroma associated with yeast cultures. In the cheese curd co-cultures described above, both the bacteria and the yeasts would have contributed to the production of esters and it was probably the yeasts that provided the alcohol substrates for ester synthesis by the bacteria (see the sections below). Moreover, ethyl acetate is one of the main volatile compounds produced by kefir yeasts (*Kluyveromyces*, *Candida*, *Saccharomyces* and *Pichia*) (Athanasiadis, Boskou, Kanellaki, & Koutinas, 2001; Kourkoutas et al., 2002).

2-Phenylethyl esters (especially phenylethyl acetate) are common esters found in surface-ripened cheeses (Roger et al., 1988; Table 1) and it is now known that yeasts are responsible for their formation. *Klu. marxianus* (var. *lactis*, var. *marxianus* and var. *fragilis*) can produce not only 2-phenylethyl acetate, but also 2-phenylethyl esters of propanoate, butanoate, isobutanoate and isovalerate (Kallel-Mhiri, Engasser, & Miclo, 1993; Fabre, Duviau, Blanc & Goma, 1995; Jiang, 1995). In addition, a range of other yeasts, including *Kloeckera saturnus*, *Hansenula anomala*, *Pic. pastoris* and *Saccharomyces delbrueckii*, can produce 2-phenylethyl acetate (Albertazzi, Cardillo, Servi, & Zucchi, 1994). We have also observed the production of 2-phenylethyl esters of acetate and propanoate by *Can. kefir* in a medium (Liu, Holland, & Crow, unpublished data), though not by *Pic. holstii* and *Deb. hansenii*.

The range of esters produced by dairy yeasts seems to be relatively limited according to the information presented above, compared with wine yeasts (*Saccharomyces* and non-*Saccharomyces*). Wine yeasts can produce a vast array of esters, including not only ethyl esters of short to medium-chain fatty acids but also acetate esters of different alcohols (Bardi, Crivelli, & Marzona, 1998; Mateo, Jiménez, Pastor, & Huerta, 2001; Rojas, Gill, Pinaga, & Manzanares, 2001). However, this may reflect the higher concentrations of alcohols (especially ethanol) in the systems studied with wine yeasts compared with the dairy systems.

Dairy yeasts have the potential to produce *S*-methylthioesters such as *S*-methylthioacetate, but the potential appears to be small and variable. For example, small amounts of *S*-methylthioacetate were produced in one study, but not in another, by the same yeasts (*Klu. lactis*, *Deb. hansenii*, *Sac. cerevisiae* and *Yar. lipolytica*) (Bonnarme, Lapadatescu, Yvon, & Spinnler, 2001b; Spinnler, Berger, Lapadatescu, & Bonnarme, 2001). It has been shown that methanethiol (a precursor to *S*-methylthioacetate) is a limiting factor for *S*-methylthioacetate synthesis in *Klu. lactis* (Arfi, Spinnler, Tache, & Bonnarme, 2002). Therefore, variable and limited production of methanethiol from methionine by yeasts (Bonnarme et al., 2001b; Spinnler et al., 2001) may explain the small and inconsistent formation of *S*-methylthioacetate by these microorganisms. The enzymes responsible for the synthesis of *S*-methylthioesters in yeasts are not defined, but could be methanethiol acyltransferases that are similar to or the same as alcohol acyltransferases (see below).

The mechanisms and enzymology of ester biosynthesis by dairy yeasts have not been fully explored, in spite of the extensive evidence of ester production by these yeasts as described above. In fact, to the best of our knowledge, there is only one report that is dedicated to the mechanism of ethyl acetate synthesis by *Klu. fragilis*. Kallel-Mhiri and Miclo (1993) demonstrated that both an esterase and an alcohol acetyltransferase were implicated in the biosynthesis of ethyl acetate by *Klu. fragilis*. The enzymes involved in the biosynthesis of esters in other dairy yeasts have not been investigated. In comparison, the enzymology of ester biosynthesis in yeasts from other sources has been well defined, and is summarised briefly below. This information may be conducive to the understanding of ester biosynthesis in dairy yeasts.

There are basically two groups of enzymes that catalyse the biosynthesis of esters in yeasts used in the fermentation of alcoholic beverages. That is, esterases and alcohol acyltransferases (especially acetyltransferases that are responsible for the biosynthesis of acetate esters). Acyl transferases are sulfhydryl enzymes that react with acetyl-CoA (and possibly acyl-CoA of a higher carbon) and alcohols to form esters. Induction of alcohol acetyltransferase activity is inhibited by aerobic conditions and unsaturated fatty acids (Malcorps, Cheval, Jamil, & Dufour, 1991). Further information on alcohol acetyltransferases of yeast can be found in a recent review by Mason and Dufour (2000).

The role that each enzyme plays varies with yeasts. In brewers', sake and wine yeasts (mainly *Sac. cerevisiae*), alcohol acetyltransferase is exclusively responsible for the biosynthesis of isoamyl acetate, whereas both

esterase and alcohol acetyltransferase catalyse the formation of ethyl acetate (Yoshioka & Hashimoto, 1981). In contrast, both esterase and alcohol acetyltransferase are responsible for the biosynthesis of isoamyl acetate in *Hansenula mrakii* (Inoue et al., 1997). The same two enzymes catalyse the formation of isoamyl acetate and ethyl acetate in *Han. anomala*, though the esterase activity is much higher than the alcohol acetyltransferase activity (Yoshioka & Hashimoto, 1981). In a strain of *Sac. cerevisiae* used in brewing, at least three enzymes (isoamyl alcohol acetyltransferase, ethanol acetyltransferase and ethanol hexanoyl-CoA transferase) are responsible for the biosynthesis of isoamyl acetate, ethyl acetate and ethyl hexanoate, respectively (Malcorps & Dufour, 1992). Esterase also catalyses the biosynthesis of ethyl esters of short to medium-chain fatty acids in *Saccharomyces* (Suomalainen, 1981; Bardi et al., 1998).

It should be noted that ester biosynthesis by alcohol acyltransferases is also operative in mammalian tissues (Kabakibi, Morse, & Laposata, 1998; Diczfalusy, Bjorkhem, Einarsson, Hillebrant, & Alexson, 2001) and fruit (Bood & Zabetakis, 2002; Shalit et al., 2001). Therefore, ester biosynthesis by alcohol acyltransferases appears to be a universal mechanism in biological systems.

As well as the esterases and alcohol acetyltransferases mentioned above, lipases can synthesise esters by esterification, as described in Section 3. Several reports have shown the biosynthesis of esters via esterification by lipases from *Can. cylindracea* (Kanisawa, 1983; Yu et al., 1992) and *Can. rugosa* (Langrand et al., 1990; Dias et al., 1991; Kim et al., 1998; Leblanc et al., 1998), although often in low-water systems. A lipase from *Can. rugosa* can catalyse ester synthesis by both esterification and transesterification (alcoholysis) under low-water conditions (Linko & Wu, 1996). Thus, the possibility of ester biosynthesis by lipases from dairy yeasts cannot be ruled out, given that many yeasts, including dairy yeasts, possess lipases (Hou, 1997; van den Tempel & Jakobsen, 2000; Addis et al., 2001; Guerzoni et al., 2001; Suzzi et al., 2001).

Recently, novel enzymes that can synthesise esters have been discovered in both methylotrophic and non-methylotrophic yeasts. In the methylotrophic yeasts (*Can. boidinii* and *Pic. methanolica*), methyl formate is synthesised from methanol and formaldehyde, catalysed by methyl formate synthase (Sakai, Murdanoto, Sembiring, Tani, & Kato, 1995; Murdanoto et al., 1997). In the non-methylotrophic yeasts (*Can. utilis* and *Sac. cerevisiae*), esters are formed from alcohols and aldehydes, catalysed by hemiacetal dehydrogenase (Kusano et al., 1998; Kusano, Sakai, Kato, Yoshimoto, & Tamai, 1999). It should be mentioned that both methyl formate synthase and hemiacetal dehydrogenase are essentially NAD^+-linked alcohol dehydrogenases, which catalyse the dehydrogenation of hemiacetals (produced non-enzymatically from alcohols and aldehydes) to form esters. It would be of interest and significance to ascertain whether dairy yeasts possess such enzymes for ester biosynthesis.

Clearly, multiple enzymes are involved in ester synthesis in yeasts. As mentioned above, the mechanism and enzymology of ester biosynthesis in dairy yeasts are largely undefined. Moreover, the physiological function of ester biosynthesis is not well understood for both dairy and non-dairy yeasts. These areas certainly warrant further research endeavour.

Alcohols are critical precursors for ester formation. Alcohol availability is generally not an issue in fermentations in which yeasts are involved. Dairy yeasts are exploited for their capability to produce alcohols (especially ethanol) in the production of alcoholic milk beverages such as kefir and koumiss (Athanasiadis et al., 2001; Kourkoutas et al., 2002), presumably from lactose, galactose and amino acids. Indeed, some dairy yeasts such as *Can. kefir* can produce 2-phenylethanol from phenylalanine (Liu, Holland, & Crow, unpublished data). Yeasts probably also convert lactate to ethanol under anaerobic conditions.

5.2. Ester biosynthesis by dairy moulds

Moulds are used as secondary cultures in the manufacture of mould-ripened cheeses (e.g., blue-veined cheese, Roquefort) and surface mould-ripened cheese (e.g., Camembert, Brie) (Gripon, 1993, 1997). The common mould cultures include *Penicillium camemberti* (and closely related *Pen. candidum*, *Pen. caseicolum*), *Pen. roqueforti* and the yeast-like mould *Geotrichum candidum* (Bockelmann, 1999). The abundance of esters in mould- and surface mould-ripened cheese (see Section 2) implies that dairy moulds are involved in ester production. However, ester biosynthesis by dairy moulds has not been thoroughly investigated.

Several researchers have investigated the production of volatile compounds including esters by dairy moulds. Jollivet, Belin and Vayssier (1993) studied the formation of volatile compounds by ten strains of *Pen. camemberti* and found that only ethyl acetate was detected among the volatiles produced by most of the strains tested. Larsen (1998) found that ethyl formate, ethyl acetate and ethyl butanoate were produced by *Pen. camemberti* and by closely related *Pen. caseifulvum*. However, no esters were found in pure cultures of *Pen. caseicolum* (Karahadian, Josephson, & Lindsay, 1985). There is no report of ester production by pure cultures of *Pen. roqueforti* from cheeses, although esters are part of the aroma components in blue-type cheese (Kinsella & Hwang, 1976). Larsen and Frisvad (1995) characterised the formation of volatile metabolites from 47 *Penicillium* taxa and found that 27 of the 47 isolates produced 28

esters (mostly acetate esters), including *Pen. camemberti* and *Pen. commune* from cheese and *Pen. roqueforti* from salami.

The yeast-like mould *Geo. candidum* can produce esters, besides *Penicillium*. A mutant of *Geo. candidum* that is known to produce a fruity aroma produced 12 esters (seven ethyl esters and five methyl propyl esters) (Latrasse, Dameron, Hassani, & Staron, 1987). In contrast, only three esters (ethyl acetate, isobutyl acetate and phenylethyl acetate) were produced by some of the eight strains of *Geo. candidum* tested (Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994). *Geo. candidum* is also known to produce a range of *S*-methylthioesters, especially *S*-methylthioacetate (Berger, Khan, Molimard, Martin, & Spinnler, 1999a; Bonnarme, et al., 2001b; Arfi et al., 2002). The high potential of *Geo. candidum* to produce *S*-methylthioesters is attributable to its ability to produce relatively large amounts of methanethiol—the critical precursor to *S*-methylthioesters (Berger et al., 1999a; Bonnarme et al. 2001a; Spinnler et al., 2001). These reports indicate that, whereas dairy moulds have the potential to produce esters, there are large species and strain variations in ester production.

The enzymology of ester biosynthesis by dairy moulds has not been thoroughly investigated. Sporadic evidence suggests that lipases from dairy moulds synthesise esters by esterification. For example, Kim et al. (1998) and Leblanc et al. (1998) reported the biosynthesis of fatty acid esters via esterification by lipases from *Pen. roqueforti*, though in solvent. Okumura et al. (1979) showed ester biosynthesis via esterification by a lipase from *Geo. candidum*. Yamaguchi and Mase (1991) reported the biosynthesis of mono- and diglyceride from glycerol and fatty acids by mono- and diacylglycerol lipase from *Pen. camemberti*. Another lipase from *Pen. camemberti* is able to synthesise ethyl valerate and ethyl butanoate via esterification in low-water systems (Leblanc et al., 1998).

Other enzymes such as alcohol acyltransferases can synthesise esters, too, besides lipases. However, there is no report of the existence in dairy moulds of alcohol acyltransferases that catalyse the biosynthesis of esters from alcohols and fatty acyl-CoAs (such as acetyl-CoA and butyryl-CoA). Nonetheless, it would be of interest to ascertain whether dairy moulds possess alcohol acyltransferases (e.g., alcohol acetyltransferase). This is because fatty acyl-CoAs (especially acetyl-CoA) are produced in abundance from fatty acid degradation in fungi (Ratledge & Dickinson, 1995). By comparison, some lipases from moulds of non-dairy origin catalyse ester synthesis via alcoholysis/transferase reaction from alcohols and acylglycerols, thus displaying acyltransferase activity (see Section 4.1).

The presence of alcohol acetyltransferases in dairy and non-dairy moulds cannot be excluded, given the abundance of acetate esters produced by the moulds mentioned above. This is supported by the fact that *Geo. candidum* can synthesise *S*-methylthioacetate from methanethiol and acetyl-CoA (Helinck et al. 2000), possibly by an alcohol acetyltransferase.

Although the presence of appropriate enzymes is important for ester biosynthesis by dairy moulds, perhaps more important is the availability of alcohols. Dairy moulds can produce a range of alcohols from a variety of substrates such as lactose, amino acids and methyl ketones (Molimard & Spinnler, 1996).

5.3. Ester biosynthesis by dairy lactic acid bacteria

LAB are used as starter cultures or as starter adjuncts to manufacture a range of fermented dairy products such as cheese and fermented milk (Stanley, 1998). LAB are also present as secondary microbial flora in fermented dairy products such as cheese (Beresford, Fitzsimons, Brennan, & Cogan, 2001; Crow, Curry, & Hayes, 2001). Although much has been published on the contribution of LAB starters to the cheese ripening process such as glycolysis, proteolysis and lipolysis (Crow, Coolbear, Holland, Pritchard, & Martley, 1993; Crow, Holland, Pritchard, & Coolbear, 1994), the role of LAB in ester biosynthesis in fermented dairy products has not been well defined.

Traditionally, *Lactococcus lactis* subsp. *lactis* strains are associated with fruity off-flavours in Cheddar cheese (Perry, 1961; Vedamuthu, Sandine, & Elliker, 1966a, 1966b) and the compounds responsible for the fruity flavour defect were identified as esters (Bills et al., 1965). However, in this case and probably with many other cheeses, the development of the fruity defect is due more to the increased production of ethanol than to the presence or absence of different LAB with different ester-synthesising activities (see below in this section). The role of LAB in ester biosynthesis was not elucidated until it was demonstrated that esterases in crude cell extracts of LAB including lactococci were capable of esterifying ethanol with butanoic and hexanoic acids to produce esters (Hosono & Elliot, 1974; Hosono, Elliot, & McGugan, 1974).

The mesophilic starters, *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, vary in their ability to produce esters. Although the two lactococcal sub-species contain similar average levels of esterase activity (Crow et al., 1994), the "*cremoris*" strains have moderately higher ethyl butanoate-synthesising activity (Liu, Holland, & Crow, 1998b). This is contrary to the traditional fruitiness associated with the "*lactis*" strains. The reason for this difference is not clear and can only be speculated upon. It is known that the "*lactis*" strains are more robust than the "*cremoris*" strains in the cheese environment, retaining greater viability and showing less autolysis (Crow et al., 1993). The "*lactis*" strains

could, therefore, retain the intracellular and physiological integrity required for the production of ethanol (Liu et al., 1998b).

The ability to form ethyl butanoate via esterification has been investigated for a large number of dairy LAB (Liu et al., 1998b). There are large species and strain differences in the potential for ethyl butanoate formation among the LAB surveyed. *Streptococcus thermophilus* strains have, on average, significantly higher ethyl butanoate-synthesising activity than other LAB. This correlates with both the high esterase activity detected in *St. thermophilus* (Crow et al., 1994) and the perceived sweet/fruity flavour notes associated with this thermophilic starter (Law, 1998). Further, the addition of *St. thermophilus* to Hispanico cheese enhanced the formation of ethyl butanoate and ethyl hexanoate as well as ethanol by up to 2.5-fold (Garde, Carbonell, Fernendez-Garcia, Medina, & Nunez, 2002).

We have observed the formation of ethyl butanoate in dry-salted Gouda cheese containing *Lactobacillus fermentum* as an adjunct (Liu, Holland, & Crow, unpublished data). There was no formation of ethyl butanoate in the cheese containing no *Lb. fermentum*. It has also been demonstrated that ethyl butanoate and ethyl hexanoate are produced in different cheeses containing different LAB including *Lb. fermentum* as adjuncts (Crow et al., 2002). By comparison, LAB from wine fermentations are also implicated in ester formation (Liu, 2002).

It should be stressed that ethanol availability is probably more critical than esterase activities in the examples of ester production by *St. thermophilus* and *Lb. fermentum* discussed above. Ethanol is known to be the limiting factor for ester formation in hard cheeses such as Cheddar and high levels of ethanol are associated with high levels of esters (Bills et al., 1965; McGugan et al., 1975; Horwood et al., 1987). Alcohols, including ethanol, are probably not the determining factor for ester production in soft cheese (mould- and surface-ripened cheese) where abundant alcohols are found (Molimard & Spinnler, 1996; Sablé & Cottenceau, 1999). LAB can produce ethanol from lactose, galactose and acetaldehyde (Fox, Lucey, & Cogan, 1990; Liu, Asmundson, Holland, & Crow, 1997). However, the ability of homofermentative LAB to produce ethanol from sugars is limited, compared with the heterofermentative LAB. LAB can also produce branched-chain alcohols from amino acids (Christensen, Dudley, Pederson, & Steele, 1999; Yvon & Rijnen, 2001).

In addition to alkyl esters, lactococci and leuconostocs can produce *S*-methylthioesters such as *S*-methylthioacetate from methanethiol and short-chain fatty acids (Lamberet, Auberger, & Bergere, 1997a). Methanethiol availability is critical for the formation of *S*-methylthioesters and this thiol is readily oxidised to sulphide compounds. Methanethiol can be produced from methionine by dairy LAB (Weimer, Seefeldt, & Dias, 1999), but usually only small amounts of this thiol can be detected due to its oxygen sensitivity.

To date, research effort has been focused on the role of esterases from LAB in ester biosynthesis. Future work should focus on enhancing the yield of ester biosynthesis via esterification, because the amount of esters produced via esterification in an aqueous enviroment is very low. For example, the esterification reaction catalysed by a lactococcal esterase gives yields of ethyl butanoate less than 2.5 ppm from 100 ppm each of substrate (butanoic acid and ethanol) and requires a freezing step at -20^{o}C for up to several days to lower water activity (Liu et al., 1998b; Nardi, Fiez-Vandal, Tailliez, & Monnet, 2002). Without a freezing step, ethyl butanoate formed was barely detectable at 18 ppb using a GC with a purge-and-trap device (Nardi et al., 2002). We have evidence that ethyl butanoate formation via esterification by esterases and whole cells of LAB in an aqueous environment (without freezing) was not detectable using GC without a purge-and-trap device (Liu, Holland, & Crow, unpublished data). There is evidence that lowering the water activity of the reaction system can increase the yield of ethyl esters of fatty acids formed via esterification catalysed by esterases of LAB (Fenster et al., 2003b).

In addition to ester biosynthesis by esterases of LAB via esterification, we have recently demonstrated, for the first time, that dairy LAB can synthesise esters in an aqueous environment from alcohols and glycerides (acylglycerols) via a transferase reaction (alcoholysis, see Section 4.1) (Liu, Holland, & Crow, 2003b). Dairy LAB vary in their ability to produce esters via alcoholysis with *St. thermophilus* and *Lb. fermentum* displaying highest transferase activities among a range of LAB studied (Liu et al., 2003b). We have further demonstrated that the enzymes that catalyse ester biosynthesis via the transferase reaction are indeed esterases that display acyltransferase activities (Liu et al., 2004).

In an aqueous environment, the yield of ester biosynthesis via alcoholysis is much higher than that of esterification (Liu et al., 2004). Therefore, we propose that alcoholysis is the major mechanism of ester biosynthesis by LAB, at least in an aqueous environment. In an environment where the water activity is low (see Section 4.2), both esterification and alcoholysis may contribute to ester formation enzymatically and non-enzymatically. The elucidation of the mechanism of ester biosynthesis by dairy LAB will certainly be conducive to controlling fruity flavour (and off-flavour) development. This can be achieved by regulating the concentration of ethanol by the use (or the avoidance) of ethanol-producing microorganisms. Alternatively, food-grade ethanol may be used.

5.4. Ester biosynthesis by dairy pseudomonads

Pseudomonads are psychrotrophs that are present as contaminants in cheese and raw milk or in pasteurised milk as a result of post-pasteurisation contamination. The research on ester production by pseudomonads has been on-going for over four decades.

Early research was focused on the production of esters by *Pseudomonas fragi* isolated from fruity flavoured Cottage cheese and pasteurised milk (Pereira & Morgan, 1958; Reddy et al., 1968; Reddy, Bills, & Lindsay, 1969b; Morgan, 1970). *Ps. fragi* is generally not regarded as an alcohol-producing bacterium, although this bacterium can produce small amounts of ethanol (Pereira & Morgan, 1958). Therefore, production of significant amounts of ethyl esters by this bacterium requires ethanol supplementation. These early investigators showed that *Ps. fragi* produced a range of esters, especially ethyl butanoate and ethyl hexanoate and that ethanol, milk fat and short-chain fatty acids were conducive to ester production by this bacterium, indicating the formation of esters by esterification. However, the mechanism of ester production by pseudomonads was not confirmed until esterases from *Pseudomonas* sp. were shown able to esterify ethanol with butanoic and hexanoic acids (Hosono & Elliot, 1974; Hosono et al., 1974). Indeed, *Ps. fragi* is strongly lipolytic and is able to hydrolyse milk fat and esterify short-chain fatty acids with ethanol (Morgan, 1976).

There has been renewed interest in the production of esters by *Ps. fragi* in the past decade or so. This is driven by a tendency world-wide toward producing natural flavours through biotechnological processes. It has been revealed that temperature and agitation speed are the physical parameters most critical to the production of esters by *Ps. fragi* (Raymond, Morin, Cormier, Champagne, & Dubeau, 1990), which may be related to the regulation (induction or repression) of esterase synthesis. Also, the production of short-chain fatty acid ethyl esters by *Ps. fragi* is dramatically enhanced by the addition of the corresponding fatty acids to the medium in the presence of ethanol (Raymond, Morin, Champagne, & Cormier, 1991). This lends further support to the notion that ester production by pseudomonads occurs via esterification (i.e., the reverse reaction of esterases). However, there is evidence that *Ps. fragi* can catalyse ester synthesis via alcoholysis, as indicated by the production of ethyl caproate from ethanol and tricaproin by this bacterium (Kermasha, Bisakowski, Morin, & Ismail, 1999). Ester production by *Ps. fragi* is also increased by about 4-fold under conditions of in situ product removal using gas stripping (Morin, Raymond, & Cormier, 1994), which would suggest product inhibition of ester biosynthesis.

Ethyl valerate is the major ester produced by *Ps. fragi* (Morin et al., 1994; Leblanc et al., 1998) and its production from ethanol and valeric acid is influenced by pH, temperature, substrate concentration and ratio, and cell biomass (Lamer, Leblanc, Morin, & Kermasha, 1996). Indeed, *Ps. fragi* has the highest esterifying activity for valeric acid and methanol or ethanol but no activity for octanoic acid (Kermasha et al., 1999; Leblanc et al., 1998). *Ps. fragi* has both lipolytic (hydrolytic) and esterifying activities; the former hydrolyses glycerides to liberate free fatty acids, whereas the latter esterifies the fatty acids with ethanol to produce esters (Kermasha et al., 1999). The hydrolytic and esterifying activities of *Ps. fragi* appear to be catalysed by two different enzymes: a lipase and an esterase. The optimal temperature for the production of both activities is different: 24–27°C for the hydrolytic activity and 15°C for the esterifying activity; similarly, the optimal assay temperature for both activities varies: 37°C for the hydrolytic activity and 12–15°C for the esterifying activity (Fonchy, Morin, Rodrigue, Muller, & Chalier, 1999). The esterifying activity of the esterase is found in the cell debris of *Ps. fragi*, which suggests that the enzyme is membrane-bound (Kermasha, Bisakowski, Ismail, & Morin, 2000).

The development of fruity off-odours (pineapple- and strawberry-like) in raw and spoiled pasteurised milks stored at refrigerated temperatures is well-recorded and is associated with growth of pseudomonads that are present naturally or that gain entry through post-pasteurisation contamination (Cormier, Raymond, Champagne, & Morin, 1991; Whitfield et al., 2000; Hayes, White, & Drake, 2002). Ester production by pseudomonads may well explain the biological origin of the fruity off-odours in the dairy products described in Section 2.1.

The salient feature of ester production by pseudomonads (e.g., *Ps. fragi*) is that the esterification reaction occurs in an aqueous milieu, as evidenced by the numerous studies described above. For comparison, the esterifying activity of a lipase from *Pseudomonas* sp. increases with increasing water activity without showing any optimum (Wehtje & Adlercreutz, 1997a, b). This process of ester production in an aqueous environment has the advantage of being natural, with no need of down-stream purification in comparison with ester biosynthesis in organic media.

From the above discussion, it becomes clear that esterases are the enzymes catalysing the biosynthesis of low molecular weight esters by pseudomonads. However, lipases from pseudomonads can also synthesise short-, medium- and long-chain esters via esterification in both organic and aqueous media (Nishio, Chikano, & Kamimura, 1988; Tan & Apenten, 1995; Tweddell, Kermasha, Combes, & Marty, 1997; Wehtje & Adlercreutz, 1997a, b). In addition to esterases and

lipases, the existence of other ester-synthesising enzymes such as alcohol acyltransferases in pseudomonads remains to be explored.

5.5. Ester biosynthesis by cheese surface bacteria

Surface (smear) bacteria, along with yeasts, are part of the microflora on the surface of surface-ripened smear cheeses such as Tilsit and Limburger. The major surface bacteria include *Arthrobacter* sp., brevibacteria, staphylococci, corynebacteria, micrococci and brachybacteria (Bockelmann, 1999; 2002; Bockelmann & Hoppe-Seyler, 2001).

The biosynthesis of esters by cheese surface bacteria has received little attention. Ethyl isovalerate was the only short-chain ester found among the volatile compounds produced by four of six brevibacteria and microbacteria studied (Jollivet, Bezenger, Vayssier, & Belin, 1992). Although cheese surface bacteria show esterolytic activities and possess esterases (Bhowmik & Marth, 1990; Rattray & Fox, 1997; Smacchi, Gobbetti, Rossi, & Fox, 2000; Curtin, Gobbetti, & McSweeney, 2002), the role of these esterases in ester biosynthesis has not been established. Nonetheless, cheese surface bacteria are likely to contribute to ester biosynthesis, considering that yeasts are prevalent among the cheese surface microflora and have the potential to produce alcohols.

A number of researchers have investigated the production of esters by corynebacteria and staphylococci from soil and fermented meats. The findings from these studies may have implications for understanding ester biosynthesis by cheese surface bacteria. *Corynebacterium* sp. isolated from soil can synthesise esters from various alcohols and fatty acids in both aqueous and organic media, although the activity is higher in organic media (Seo, Yamada, & Okada, 1982). Staphylococci used in meat fermentation can esterify ethanol with short to medium-chain fatty acids to produce ethyl esters in aqueous media (Talon, Chastagnac, Vergnais, Montel, & Berdague, 1998), suggesting the involvement of esterases. It has also been revealed that immobilised staphylococcal lipases can esterify ethanol with fatty acids of up to C18 in solvent (Talon, Rouchon, Denoyer, Montel, & Berdague, 1995; Talon, Montel, & Berdague, 1996).

Cheese surface bacteria are known for their ability to produce *S*-methylthioesters. For instance, brevibacteria and micrococci can produce *S*-methylthioacetate in culture media (Cuer, Dauphin, Kergomard, Dumont, & Adda, 1979a,b). Further, brevibacteria, corynebacteria and micrococci can esterify methanethiol with a variety of short-chain fatty acids to produce *S*-methylthioesters of up to C6 (Lamberet et al., 1997a; Lamberet, Auberger, & Bergere, 1997b). *Arthrobacter* sp. and *B. linens* are the strongest *S*-methylthioester producers among the cheese surface bacteria examined by Bonnarme et al. (2001b).

The enzymes involved in *S*-methylthioester biosynthesis in cheese surface bacteria are presumably acyltransferases and esterases, which catalyse the biosynthesis of *S*-methylthioesters from methanethiol and fatty acyl-CoAs or from methanethiol and fatty acids, respectively. The amount of *S*-methylthioesters produced is likely to be dependent upon the availability of the critical precursor-methanethiol, which can be produced from methionine by dairy microorganisms including yeasts and cheese surface bacteria (Bonnarme et al., 2001b).

5.6. Ester biosynthesis by propionibacteria

Propionibacteria are used as adjunct cultures in the manufacture of Swiss-type cheese (Noël et al., 1999). Several propionate esters and other alkyl esters are found in this type of cheese (see Section 2). Propionibacteria have the potential to form ethyl butanoate by esterifying ethanol with butanoic acid (Liu et al., 1998b), which is supported by the presence of esterases in these bacteria (Dupuis & Boyaval, 1993; Dupuis, Corre, & Boyaval, 1993; Suoniemi & Tynkkynen, 2002). Several branched-chain esters are found in Swiss-type cheeses, such as ethyl 3-methylbutanoate, 3-methylbutyl acetate, 3-methylbutyl propanoate and 2-methylbutyl acetate (Thierry & Maillard, 2002). The formation of branched-chain esters is likely to be related to the catabolism of branched-chain amino acids by propionibacteria (strain-dependent) (Thierry & Maillard, 2002). In addition, propionibacteria are implicated in the formation of *S*-methylthioesters such as *S*-methylthioacetate and *S*-methylthiopropanoate in Swiss-type cheeses, but again this appears to be strain-dependent (Thierry & Maillard, 2002).

Esterases of propionibacteria may be at least partially responsible for the biosynthesis of esters. However, alcohol acyltransferases may be the enzymes that catalyse the biosynthesis of propionate esters (and other esters), given that propionyl-CoA would have been produced abundantly during growth and metabolism of propionibacteria. Nonetheless, the presence of alcohol acyltransferase in propionibacteria has not been reported. It will be worthwhile to verify whether esterases of propionibacteria are indeed alcohol acyltransferases, as demonstrated with LAB (Section 5.3), so that the mechanism of ester biosynthesis in propionibacteria can be elucidated.

6. Ester hydrolysis

The primary impact of esterases and lipases in relation to lipid metabolism in dairy systems is probably

the hydrolysis of esters including acylglycerols (tri-, di- and mono-). Therefore, it is not surprising to note that the concentration of esters may decrease during storage of fermented dairy products. Indeed, decreases in the concentration of some esters have been observed during ripening of some cheese such as Grana Padano and Serra da Estrela (Moio & Addeo, 1998; Dahl et al., 2000), suggesting the hydrolysis of esters.

Lipases are known to hydrolyse acylglycerols (tri-, di- and mono-) containing fatty acids of both long- and short-carbon chain lengths. Bacterial esterases can hydrolyse tri-, di- and mono-acylglycerols containing fatty acids of short to medium-carbon chain lengths. Examples of the latter include esterases from *Lb. plantarum* and *St. thermophilus* (Gobbetti, Fox, & Stepaniak, 1997; Liu, Holland, & Crow, 2001). In addition to the hydrolysis of acylglycerols, lipases and esterases can hydrolyse alkyl esters, including those esterases and lipases from LAB, pseudomonads, moulds and yeasts (Suomalainen, 1981; Nakagawa, Tsujita, & Okuda, 1984; Wehtje & Adlercreutz, 1997a, b; Fenster, Parkin, & Steele, 2003a). Wine LAB are implicated in the hydrolysis of alkyl esters (du Plessis, Steger, du Toit, & Lambrechts, 2002). In *S. cerevisiae*, the balance of alcohol acetyltransferase and esterase activities is important for isoamyl acetate accumulation because of the ability of esterase to hydrolyse esters (Fukuda et al., 1998).

Ester accumulation is the difference between ester synthesis and ester hydrolysis; where the equilibrium set is dependent upon the environmental conditions (water activity, availability of substrates, etc.). Net ester accumulation is determined by a dynamic balance between ester synthesis and ester hydrolysis. Further work is required to study ester hydrolysis so as to maximise ester synthesis or to minimise ester hydrolysis, being dependent upon whether fruitiness is a defect or not.

7. Metabolic engineering and future research

With the advent of molecular biology, various strategies have been applied to LAB to genetically engineer strains that can over-produce certain metabolites with industrial importance such as polysaccharides, diacetyl, ethanol and mannitol. Common approaches include gene over-expression and inactivation. A great deal of information is available on the metabolic engineering of LAB of dairy origin, *Lc. lactis* in particular (de Vos, 1996; de Vos et al., 1998; Hugenholtz & Kleerebezem, 1999; Kleerebezem, Hols, & Hugenholtz, 2000; Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2001; Hoefnagel et al., 2002). In contrast, relatively little is known about the application of metabolic engineering to yeasts and moulds of dairy and non-dairy origin for the production of industrially important compounds. The exception is *Sac. cerevisiae* and a great deal of information is available about the genetics, molecular biology and metabolic engineering of this industrially important yeast (Fukuda et al., 1998; Mason & Dufour, 2000; Olsson & Nielsen, 2000; Pretorius, 2000, 2003; Overkamp et al., 2002; Pretorius & Bauer, 2002).

Metabolic engineering has not been applied to dairy microorganisms for the production of esters, although this approach has great potential for controlling ester biosynthesis in fermented dairy products. The gene targets for metabolic engineering are genes encoding esterases, lipases and alcohol acyltransferases. Over-expression of genes encoding these enzymes may enhance ester production, provided that alcohols are available. An example of this is the over-expression of a tributyrin esterase in *Lc. lactis* (Fernández et al. 2000) and correspondingly, the synthesis of esters by the enzyme is increased (Liu et al., 2004). Conversely, inactivation of genes encoding these enzymes may eliminate ester formation, regardless of alcohol availability. An example of this is the construction of an esterase negative mutant of *Lc. lactis* and correspondingly, the formation of esters is reduced (Nardi et al., 2002). Ethanol production by metabolic engineering of homofermentative LAB such as *Lc. lactis* is of practical importance, because ethanol is often the limiting factor for ester production in hard cheeses and fermented milks in which *Lc. lactis* is the primary starter.

Metabolic engineering has been successfully exploited to increase ester biosynthesis by *Saccharomyces* yeast and non-dairy bacteria. Lilly, Lambrechts and Pretorius (2000) over-expressed the alcohol acetyltransferase genes of *Sac. cerevisiae* in commercial wine yeasts and observed increased production of several acetate esters by up to 12-fold in wines fermented by the modified yeasts. Similarly, Verstrepen et al. (2003b) found that the expression levels of the alcohol acetyltransferase genes of *Sac. cerevisiae* control the synthesis of esters in brewing yeasts. Another interesting approach is the expression of the alcohol acetyltransferase genes of *Sac. cerevisiae* in industrially important, solvent-producing *Clostridium acetobutylicum* (Horton, Huang, Bennett, & Rudolph, 2003). Chang, Chou and Shaw (2001) obtained recombinant *Staphylococcus epidermis* lipases with increased activity and improved substrate specificity for ester biosynthesis via esterification in an aqueous environment. These examples illustrate a potential for the application of metabolic engineering to dairy microorganisms to increase ester production in fermented dairy products.

Esterases can also hydrolyse alkyl esters, as described above (see Section 6). Thus, metabolic engineering has also been exploited to decrease the level of esters during ethanolic fermentation when esters become undesirable

by-products. Hasona, York, Yomano, Ingram and Shamugam (2002) cloned an esterase gene from a strain of *Ps. putida* and expressed the esterase gene in ethanologenic *Escherichia coli* KO11. The recombinant *E. coli* KO11 containing the esterase gene was able to reduce the level of ethyl acetate by up to 10-fold during ethanolic fermentation. This is surprising, considering that many pseudomonads can catalyse ester synthesis by esterification (see Section 5.4). Presumably, this esterase did not have the ability to synthesise esters by esterification. This molecular approach to ester reduction has implications for dairy fermentation where fruitiness may be an off-odour.

8. Conclusions

Esters constitute an integral part of the cheese aroma array and play an important role in the cheese flavour balance. Dairy microorganisms (bacteria, moulds and yeasts) are the primary producers of esters. Ester biosynthesis is catalysed by esterases, lipases and alcohol acyltransferases. Esterases and lipases may also hydrolyse esters. Water activity and the availability of alcohols are the two determining factors of ester biosynthesis. An understanding of ester biosynthesis will help control ester production in fermented dairy products. Metabolic engineering is expected to have a significant impact on ester biosynthesis by dairy microorganisms.

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