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International Journal of Food Microbiology 78 (2002) 79–97

INTERNATIONAL JOURNAL OF
Food Microbiology

www.elsevier.com/locate/ijfoodmicro

Food spoilage—interactions between food spoilage bacteria

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Accepted 26 May 2002

Abstract

Food spoilage is a complex process and excessive amounts of foods are lost due to microbial spoilage even with modern day preservation techniques. Despite the heterogeneity in raw materials and processing conditions, the microflora that develops during storage and in spoiling foods can be predicted based on knowledge of the origin of the food, the substrate base and a few central preservation parameters such as temperature, atmosphere, a_w and pH. Based on such knowledge, more detailed sensory, chemical and microbiological analysis can be carried out on the individual products to determine the actual specific spoilage organism. Whilst the chemical and physical parameters are the main determining factors for selection of spoilage microorganisms, a level of refinement may be found in some products in which the interactive behavior of microorganisms may contribute to their growth and/or spoilage activity. This review gives three such examples. We describe the competitive advantage of *Pseudomonas* spp. due to the production of iron-chelating siderophores, the generation of substrates for spoilage reactions by one organism from another microorganism (so-called metabiosis) and the up-regulation of phenotypes potentially involved in spoilage through cell-to-cell communication. In particular, we report for the first time the widespread occurrence of *N*-acyl homoserine lactones (AHL) in stored and spoiling fresh foods and we discuss the potential implications for spoilage and food preservation.
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Keywords: *N*-Acyl homoserine lactones; Antagonism; Food spoilage; Metabiosis; Quorum sensing; Siderophores; Specific spoilage organisms

1. Introduction

Preservation of foods has, since the beginning of mankind, been necessary for our survival. The preservation techniques used in early days relied—without any understanding of the microbiology—on inactivation of the spoiling microorganisms through drying, salting, heating or fermentation. These methods are still used today, albeit using less and less preservation and combining various lightly preservation procedures to inhibit growth of microorganisms. Spoilage is characterised by any change in a food product that renders it unacceptable to the consumer from a sensory point of view. This may be physical damage, chemical changes (oxidation, colour changes) or appearance of off-flavours and off-odours resulting from microbial growth and metabolism in the product. Microbial spoilage is by far the most common cause of spoilage and may manifest itself as visible growth (slime, colonies), as text-

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URL: http://www.dfu.min.dk/micro/lg.htm.

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PII: S0168-1605(02)00233-7

ural changes (degradation of polymers) or as off-odours and off-flavours. Despite chill chains, chemical preservatives and a much better understanding of microbial food spoilage, it has been estimated that 25% of all foods produced globally is lost *post harvest* or *post slaughter* due to microbial spoilage (Anonymous, 1985).

This review gives a brief introduction to the specific spoilage organism concept and an overview of what is currently known about spoilage microbiology of different food products. The microorganisms dominating a product can be predicted and qualified guesses can be made in terms of the organisms causing the spoilage by understanding how the major preservation parameters affect microbial selection. Clear patterns emerge, independent of raw material and processing, in terms of which organisms dominate and spoil food products as a function of, e.g. origin, substrate base, temperature, pH, a_w and atmosphere. We believe that such understanding of the microbial ecology is essential if the excessive *post harvest* and *post slaughter* losses are to be reduced. Also, knowledge of the microorganisms involved in spoilage and the metabolites associated with spoilage is needed to develop microbiological and chemical methods for evaluation of quality and shelf life.

The growth and activity of spoilage microorganisms is mostly described and studied as function of substrate base and of chemical and physical parameters such as temperature, pH, a_w and atmosphere. The importance of these conditions for the selection of a spoilage microflora cannot be underestimated, however, it is becoming increasingly clear that in some situations also interactive behaviour between the microorganisms determines selection and/or metabolism and subsequently spoilage. The major part of this review gives 3 examples of microbial interactive behaviour of potential importance in microbial food spoilage, namely (i) antagonism, (ii) metabiosis and (iii) cell-to-cell communication.

2. The concept of specific spoilage organisms

Each and every food product harbours its own specific and characteristic microflora at any given point in time during production and storage. This microflora is a function of raw material flora, processing, preservation and storage conditions. Despite the variability in all of the three, some very clear patterns emerge, and based on knowledge of a few chemical and physical parameters it is possible with great accuracy to predict which microorganisms will grow and dominate in a particular product. At the point of sensory rejection (spoilage), the so-called spoilage microflora (or spoilage association) is composed of microorganisms that have contributed to the spoilage and microorganisms that have grown but not caused unpleasant changes. The former is the so-called specific spoilage organism(s) (SSO) of the product.

The spoilage potential of a microorganism is the ability of a pure culture to produce the metabolites that are associated with the spoilage of a particular product. In general, several of the organisms isolated from a food product will be able to produce spoilage metabolites when allowed unlimited growth. It is crucial that quantitative considerations are introduced (Gram, 1989) since the spoilage activity of an organism is its quantitative ability to produce spoilage metabolites (Dalgaard et al., 1993; Dalgaard, 1995). Thus it is to be evaluated if the levels of the particular organism reached in naturally spoiling foods are capable of producing the amount of metabolites associated with spoilage. In general, it requires a careful combination of microbiology, sensory analyses and chemistry to determine which microorganism(s) are the SSOs of a particular food product.

Despite the importance of microorganisms in food spoilage, the definition and assessment of spoilage relies on sensory evaluation. As outlined above, specific microorganisms are the cause of spoilage, however, neither the level of "total count" of, e.g. 10^7 cfu/cm^2 (Mano et al., 1995) nor the level of SSO per se can directly predict the sensory quality of a product. In contrast, the level of SSO can be used to predict remaining shelf life of a product under conditions where the SSO is important. Examples of this is the prediction of remaining iced shelf life of cod based on numbers of *Shewanella putrefaciens* (Jørgensen et al., 1988) and of modified atmosphere packed cod based on numbers of *Photobacterium phosphoreum* (Dalgaard et al., 1997).

3. Food spoiling microorganisms

Almost all groups of microorganisms harbour members that under some conditions can contribute to spoilage of foods. Theoretically, one can assume that

all microorganisms are initially present on a food product where after a selection occurs—based primarily on nutrient composition and on the chemical and physical parameters. Similar microfloras emerge in different food products under the same conditions despite the heterogeneity in the outset. Thus *Pseudomonas* spp. and a few other Gram-negative psychrotrophic organisms will dominate proteinaceous foods stored aerobically at chill temperatures. This is true for meat, poultry, milk and fish. If pH is high like in fish and "Dark Firm Dry" (DFD) meat, *S. putrefaciens*-like organisms develop in parallel, and may become the dominant spoilage organisms, as is the case in marine, iced fish (Chai et al., 1968). The pseudomonads in pasteurised milk originate from post-process contamination (Eneroth et al., 2000) but the product may also spoil due to growth of psychrotrophic *Bacillus* (Ternström et al., 1993) of which the spores have survived the heat treatment.

In meat and fish, a change in atmosphere, e.g. by vacuum-packing, will inhibit the respiratory pseudomonads and in meats cause a shift in the microflora to lactic acid bacteria (LAB), Enterobacteriaceae and sometimes *Brochothrix thermosphacta* (Dainty and Mackey, 1992). Also, clostridia may cause a deep-muscle anaerobic spoilage of vacuum-packed meats (Broda et al., 1996). *S. putrefaciens*, which is capable of anaerobic respiration, also grows and contributes to spoilage in meat with high pH. In fish, vacuum packaging selects for *S. putrefaciens* and for the CO_2-resistant, psychrotolerant marine bacterium *P. phosphoreum*. CO_2 packaging of fish from temperate waters does not select for LAB but allows *P. phosphoreum* to grow and this organism spoils the product (Dalgaard et al., 1993). A mild heat treatment of fish results in elimination of vegetative bacteria and clostridia and *Bacillus* (that both survive as spores) may grow and spoil the product, especially if vacuum-packed (Ben Embarek, 1994).

Further "selective pressure", e.g. addition of low levels of salt to and drying of fish eventually switch the microflora in the same direction as vacuum-packed meat and meat products. Thus a microflora of LAB, Enterobacteriaceae and—to some extend—*Brochothrix* develops (Lyhs et al., 1998; Truelstrup-Hansen and Huss, 1998; Joffraud et al., 2001). Due to the aquatic origin, *P. phosphoreum* is often also present. Identifying the SSO of lightly preserved fish products and of processed meat products has proven difficult and probably different groups of bacteria are important under different conditions. Thus in some trials, the indicators of spoilage parallels the metabolites of *P. phosphoreum*, in other trials that of a LAB-flora (*Lactobacillus curvatus*) and in others the combined metabolites of LAB and Enterobacteriaceae (Jørgensen et al., 2000a,b).

Increasing the preservation by a decrease in pH (below 5), an increase in the NaCl concentration (above 6%) and by adding sorbate and/or benzoate eliminates the Gram-negative microflora. LAB and yeast are the remaining organisms in semi-preserved fish products. Different *Lactobacillus* spp., including *Lactobacillus alimentarius* (Lyhs et al., 2001) have been identified as spoilage organisms of these products. Meats that are cured by salt and low pH are usually shelf-stable and are not spoiled by microbial growth (ICMSF, 1998). The preservation profile of, e.g. mayonnaise-based salads is similar to the semi-preserved fish products in terms of pH, temperature, atmosphere and chemical preservatives and a microflora of LAB may develop (Delaquis et al., 1997; ICMSF, 1998). Also, fruit juices, which are high in sugar and have a low pH (typically between 2.0 and 4.5), often spoil due to growth of LAB and/or yeasts (Edwards et al., 1998; Tajchakavit et al., 1998). In pasteurised juice, the acid tolerant spore forming bacteria *Alicyclobacillus acideoterrestris* is a major spoilage organism (Walls and Chuyate, 2000). Decreasing a_w further eliminates bacterial growth, and only extremophiles (such as halophiles or osmophiles) and filamentous fungi are capable of developing on dried, salted fish, meat and fruit products (Pitt and Hocking, 1997). Thus, as the preservation profile increase in inhibitory strength, the microflora changes along the following path: non-fermentative psychrotrophic Gram-negative bacteria → fermentative Gram-negative bacteria → LAB → yeasts → filamentous fungi.

Food products of vegetable origin presents a special case due to the nutrient composition. The high pH will allow a range of Gram-negative bacteria to grow, but spoilage is specifically caused by organisms capable of degrading the vegetable polymer, pectin (Liao, 1989; Liao et al., 1997). These organisms, typically *Erwinia* and *Pseudomonas* species are the SSO of several ready-to-eat vegetable products

(Nguyen-The and Prunier, 1989; Lund, 1992) but also pectin degrading fungi can play a role in vegetable spoilage (Pitt and Hocking, 1997).

The presence of ethanol, as in beer and wine, is similar in "preservation strength" to, e.g. low pH and salted foods and only allows LAB and yeasts to grow. Resistance to the antibacterial compounds found in hop is required for an organism to grow in beer. *Lactobacillus* species are tolerant to these iso-α-acids and several species have been identified as SSOs in beer (Sakamoto et al., 2001). Also, several *Pediococcus* species are important beer spoiling organisms (Jespersen and Jakobsen, 1996).

Examples of the influence of the "preservation pressure" on the selection of spoilage microflora in a range of food products are shown in Table 1.

3.1. Food spoiling reactions

As mentioned, microbial spoilage usually manifests itself as slime and/or off-odours and off-flavours. Many studies have described in more chemical terms what compounds characterise the spoilage of particular products and which substrates have been used by the microorganisms to form these compounds (Table 2). Such knowledge is important, e.g. if a chemical

Table 1
Typical spoilage organisms of food products depending on physical/chemical preservation profile

Temperature		Atmosphere		pH		a_w		Substrate-base			Typical product	Typical spoilage organism
Low	High	Aerobic	Non-aerobic	Low	High	Low	High	Amino acids	Simple CHO	Complex CHO		
x		x			x		x	x			fish	*Shewanella*, *Pseudomonas*
x			x		x		x	x			fish	*Photobacterium*, *Shewanella*
x			x		x	(x)		x			smoked fish	LAB, Enterobacteriaceae, *Photobacterium*
x			x	x		(x)		x	(x)		marinated fish	LAB, yeasts
x		x		x			x	x	(x)		meat	*Pseudomonas*
x			x	x			x	x	(x)		meat	LAC, Enterobacteriaceae, *Brocothrix*, clostridia
x			x	(x)		(x)		x	(x)		meat products	LAC, Enterobacteriaceae, *Brochotrix*
x		x			x		x		x		milk	*Pseudomonas*, *Bacillus*
	x	x			x		x			x	raw vegetables	*Erwinia*, *Pseudomonas*, fungi
	x		x		x		x	x			eggs	*Pseudomonas*, Enterobacteriaceae
	x	x		x			x		x	(x)	fruits	yeasts, filamentous fungi
x			x	x		x			(x)		mayonnaise salads	yeasts, LAB
	x		x	x			x		(x)		beer	LAB, yeasts
	x		x	x			x		(x)		wine	LAB, yeasts
	x	x			x	x				x	cereals	filamentous fungi
	x	x			x	x				x	nuts	filamentous fungi

Table 2
Examples of typical spoilage substrates and metabolites found in microbiologically spoiled foods

Sensory impression	Spoilage product	Spoilage substrate	Food Product	Specific spoilage organism	Reference
Slime	EPS (dextran)	sucrose	kimchi	*Leuconostoc*	Kim et al. (2001)
			turkey breast	*Leuconostoc*	Samelis et al. (2000)
		sugars	wine	*Pediococcus damnosus*	Walling et al. (2001)
		sugars	bread	*Bacillus*	Thompson et al. (1998)
Slime	hydrolysed polymer	pectin	vegetables	*Erwinia*, *Pseudomonas*	Nguyen-The and Prunier (1989)
Fishy off-odour	trimethylamine (TMA)	trimethylamine oxide (TMAO)	fish	*S. putrefaciens*	Shaw and Shewan (1968)
				P. phosphoreum	Dalgaard et al. (1993)
				Aeromonas spp.	Gram et al. (1990)
Ammonia, putrid	NH_3	amino acids	proteinaceous foods	many microorganisms	
	Biogenic amines	amino acids	meat	Enterobacteriaceae and LAB	Edwards et al. (1985)
			fish[a]	Enterobacteriaceae, LAB	Jørgensen et al. (2000b)
			fish[a]	*P. phosphoreum*	Jørgensen et al. (2000b)
Sulphidy off-odour	H_2S	cysteine	fish, meat	*S. putrefaciens*	Chai et al. (1968), Herbert and Shewan (1976)
				Enterobacteriaceae *L. saké*, *L. curvatus*	Dainty and Mackey (1992)
Greening	H_2S	cysteine	meat	*L. plantarum*	Lee and Simard (1984)
Sulphydryl off-odours	$(CH_3)_2S_2$	methionine	fish, meat	*Pseudomonas* spp. Enterobacteriaceae	Segal and Starkey (1969), Herbert and Shewan (1975)
Acid off-odour	acetic acid L,D-lactic acid	glucose, ribose, other CHO	meat	LAB	Nassos et al. (1983)
"Sweet curdling"	proteinaceous fat particles	phospholipid	milk	*B. cereus*	IDF (1992)
Fruity off-odour	esters		fish	*P. fragi*	Miller et al. (1973)
			milk	*P. fragi*, *P. putida* and *Y. intermedia*	Cormier et al. (1991), Whitfield et al. (2000)
Cheesey off-odour	acetoin, diacetyl, 3-methylbutanoyl	glucose	meat	*B. thermosphacta* Enterobacteriaceae homofermentative LAB	Dainty and Mackey (1992)
Medicine off-odour	2-methoxy-phenol, sediment	sugars	juice	*A. acidoterrestris*	Walls and Chuyate (2000)
Musty odour	trichloroanisol	2,4,6 trichlorophenol	wine	*P. brevicompactum*, *A. flavus*	Filtenborg et al., 1996

[a] Biogenic amines may not be the cause of spoilage but can serve as a spoilage index (Jørgensen et al., 2000a).

spoilage index is to be developed. Examples of this include trimethylamine of iced, marine fish. This knowledge may also be used to eliminate a particular spoilage promoting compound, e.g. sucrose as sweetener in brined shrimp. In this case, slime/ropiness is formed by *Leuconostoc* from sucrose and substituting with, e.g. artificial sweeteners or sugar alcohols, may prevent this spoilage (From, 1988).

4. Interactions between food spoiling bacteria

The selective conditions imposed on the food microbial community by physical and chemical parameters, as outlined in Table 1, undoubtedly are the most important in terms growth and selection of microorganisms. However, microbial food spoilage is a process involving growth of microorganisms to numbers (10^7-10^9 cfu/g) at which the microorganisms also must be assumed to interact and influence the growth of one-another (Boddy and Wimpenny, 1992). The interactions between microorganisms may be classified on the basis of their effects as being detrimental or beneficial (Fredrickson, 1977). Several types of interactions have been studied in food ecosystems including both antagonistic and coordinated behaviour and interactions where growth or a particular metabolism of one organism is favoured by the growth of another organism. This section gives three examples of such behaviour:

- antagonism caused by the competition for iron as mediated by bacterial siderophore production and subsequent suppression of maximum cell density of less competitive bacteria,
- change in spoilage profile of an organism by the supply of nutrients from another microorganism (metabiosis),
- the ability of Gram-negative bacteria to coordinate the expression of certain phenotypic traits (e.g. hydrolytic enzymes) through bacterial communication via *N*-acyl homoserine lactones (AHLs).

4.1. Antagonism

Changes in environmental conditions, e.g. by lowering of pH, can be a powerful way for a microorganism to antagonise other bacteria and create a selective advantage. Also, competition for nutrients may select for the organisms best capable of scavenging the limiting compound(s). Several microorganisms important in food spoilage have such antagonistic abilities. Thus, the lactic acid bacteria cause a lowering of pH and may produce antibacterial peptides (bacteriocins) (Adams and Nicolaides, 1997). The spoilage reactions of certain Gram-negative bacteria may produce NH_3 and trimethyl-amine, which are toxic to a number of other bacteria and sometimes to the producing organism itself. *Pseudomonas* spp., in particular the fluorescent group, produce a range of antibacterial and antifungal compounds such as antibiotics and cyanide, and at the same time they compete very efficiently for iron (Ellis et al., 2000).

4.1.1. Competition for iron

Despite the richness of nutrients in most foods, bacteria may be limited in selected compounds such as minerals, amino acids or sugars. Thus, it has been shown that the concentration of iron is low in several fish products (Gram, 1994). Iron is essential for most microorganisms and is used in bacterial respiration (as electron shuttler) and in redox enzymes. Due to the high oxidative power of Fe^{3+}, iron is mostly bound in insoluble complexes in the environment and in mammals and plants. Most microorganisms have therefore developed highly specific iron chelating systems, and often they produce siderophores, which are iron-chelators secreted by the cell. Upon binding of iron the siderophore-iron complex is taken up by the microbial cell and iron is liberated internally (Crosa, 1997). In particular, pseudomonads are prominent producers of siderophores with high iron binding constants. The iron chelating ability has been of particular interest in the rhizosphere, where the use of pseudomonads as biocontrol agents against fungal diseases has been attributed in part to their competitive advantage vis-a-vis iron (O'Sullivan and O'Gara, 1992; Ellis et al., 2000).

Bacteria growing on fish produce siderophores (Gram and Melchiorsen, 1996) that are only induced when the iron concentration is limiting. Siderophores have been extracted from cheeses indicating that this food is iron limited (Ong and Nielands, 1979). Also, bacteria spoiling eggs must be able to scavenge iron from the tight iron binding proteins of the egg (cf. Cox, 2001). *Pseudomonas* spp. isolated from foods produce siderophores (Gram, 1993; Cheng et al., 1995; Laine et al., 1996), in particular strains isolated from fish (Gram, 1993; Champomier-Vergès and Richard, 1994). Also *S. putrefaciens* chelates iron by siderophore production (Gram, 1994), but despite this ability, it is strongly inhibited by siderophore producing pseudomonads under iron-limited conditions (Gram, 1993). When grown in co-culture on fish samples, siderophore producing pseudomonads inhibits, e.g. *Shewanella* when the former reach approximately 10^8 cfu/g and cause a

suppression of the maximum cell density of the inhibited organism (Gram and Melchiorsen, 1996; Fig. 1). The depression of maximum cell density of microorganisms by an "over growing" microflora is also seen for *Listeria monocytogenes* growing in lightly preserved foods with a dominant lactic acid bacterial flora (Grau and Vanderlinde, 1992; Nilsson et al., 1999). The latter phenomenon may be caused by bacteriocin production by the LAB, but since several non-bacteriocin producing LAB are as inhibitory (Nilsson et al., 1999), the inhibition may as well be explained by the LAB out competing the *Listeria* on a few essential nutrients (Buchanan and Bagi, 1997). This suppression (of maximum population density) of a

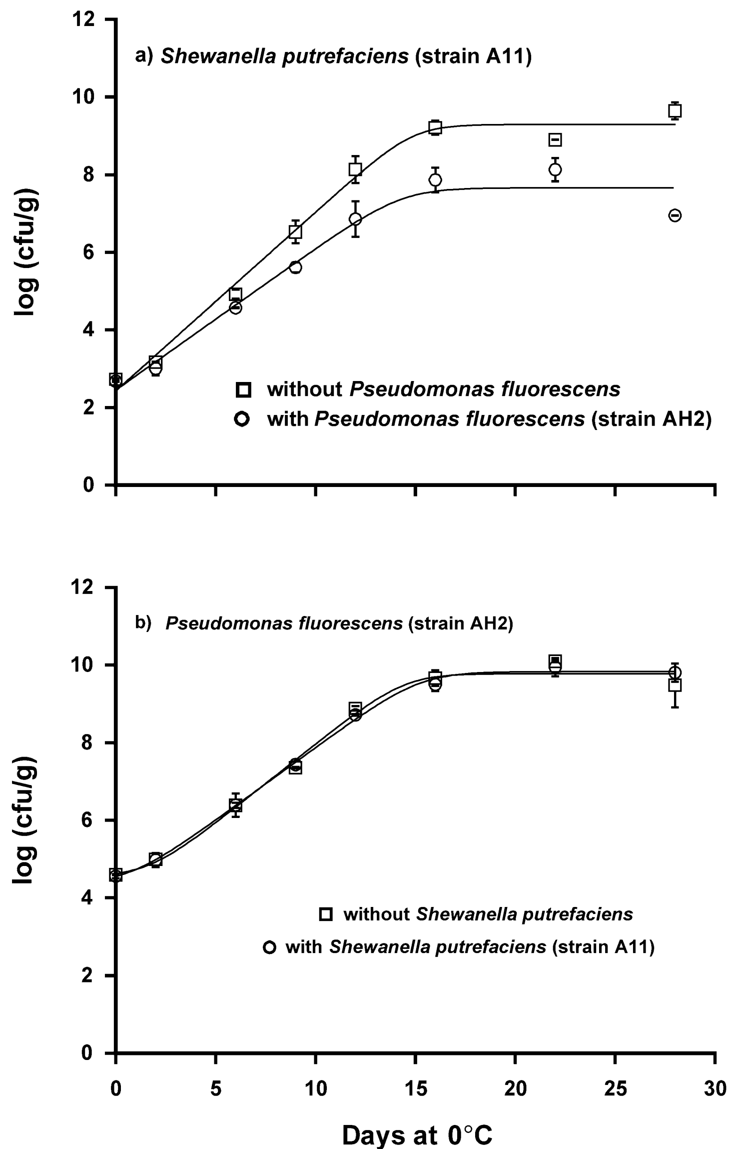

Fig. 1. The suppression of maximum cell density of *S. putrefaciens* by siderophore producing *P. fluorescens* when grown on cod muscle at 0 °C (modified from Gram and Melchiorsen, 1996).

particular organism by an overgrowing microflora is called the Jameson effect (Jameson, 1962; Stephens et al., 1997; Ross et al., 2000).

It is not clear to what extend the competitive activity of, e.g. pseudomonads contribute to their dominance during spoilage of proteinaceous foods. Since *S. putrefaciens* can be isolated from fresh Nile perch and the fish contains trimethylamine oxide, it would be expected that this bacterium contributed to spoilage of the iced fish. However, spoilage is exclusively caused by *Pseudomonas* spp. (Gram et al., 1990) and it has been suggested that their competitive ability contributes to this dominance (Gram and Melchiorsen, 1996).

Removal of iron and other trace metals has been suggested as a way of controlling growth of spoilage microorganisms. Spoilage yeasts did not grow in grape juice in which iron was chelated by resins (Feng et al., 1997). It is, however, unlikely that addition of high concentrations of iron chelators will be widely adopted due to the obvious negative effects on the nutritional value of food products limited in iron.

4.2. Metabiosis

Innumerable ways of inter-dependency exists between different organisms. The term metabiosis describes the reliance by an organism on another to produce a favourable environment. This can be the removal of a oxygen by a Gram-negative microflora allowing anaerobic organisms such as *Clostridium botulinum* to grow (Huss et al., 1979) or it can be situations where one organism provide nutrients enhancing growth of another. Thus several studies have shown that despite the inhibitory activity of pseudomonads as described above, their presence may also enhance growth of some microorganisms. Pre-inoculation of milk with different Gram-negative psychrotrophic bacteria subsequently yielded higher growth and more acid from lactic acid bacteria (Cousin and Marth, 1977) and growth of *Staphylococcus aureus* may also be stimulated by *Pseudomonas* spp. (Seminiano and Frazier, 1966). Such nutrient inter-dependency may also play a role in food spoilage (Dainty et al., 1986; Jørgensen et al., 2000b).

Biogenic amines, which are formed by bacterial decomposition of amino acids, have been suggested as a quality indicator of vacuum-packed beef (Edwards et al., 1985). The spoilage microflora of this product typically consists of a mixture of LAB and Enterobacteriaceae. *Hafnia alvei* and *Serratia liquefaciens* (today: *Serratia proteamaculans*) as single cultures produce levels of cadavarine similar to the naturally contaminated product, however, the level of putrescine from single cultures does not correspond to the spoiling product. Some LAB degrade arginine to ornithine (which is the pre-cursor of putrescine) and co-inoculation of the putrescine-forming Enterobacteriaceae with arginine-degrading LAB resulted in a 6–15 time greater production of putrescine than in single cultures (Dainty et al., 1986; Table 3). The changes in biogenic amines and pH during chill-storage of vacuum-packed cold-smoked salmon can be combined to a quality index that correlates with sensory evaluation of the product (Jørgensen et al., 2000a). High levels of biogenic amines may be produced by single cultures of *P. phosphoreum* or by *L. curvatus* (Jørgensen et al., 2000b) but may also arise from co-cultures of lactobacilli and Enterobac-

Table 3
Metabiotic interaction between lactic acid bacteria and Enterobacteriaceae isolated from spoiled vacuum-packed meat (modified from Edwards et al., 1985; Dainty et al., 1986)

Product	Bacterial strain/species present						
	S. liquefaciens (cfu/g)	*H. alvei* (cfu/g)	Enterobacteriaceae (cfu/g)	arginine-degrading LAB (cfu/g)	LAB (cfu/g)	Putrescine (μg/g)	Cadaverine (μg/g)
Meat	$10^5–10^8$	–	–	–	–	1	58
Meat	10^5	–	–	3×10^7	–	20	59
Meat	–	10^7	–	–	–	4	58
Meat	–	10^6	–	10^7	–	32	58
Meat	–	–	–	$10^7–10^8$	–	1	< 0.1
Spoiling meat	–	–	$5 \times 10^4–10^6$	–	$10^6–10^7$	15–20	50

teriaceae. Similar to the studies from vacuum-packed meat (Dainty et al., 1986), production of putrescine was enhanced 10–15 times when ornithine-decarboxylase-positive Enterobacteriaceae were co-cultured with arginine-deiminase positive LAB as compared to single cultures (Jørgensen et al., 2000b). Other studies have similarly found that interaction between LAB and Enterobacteriaceae can be important in food spoilage. Borch et al. (1996) found that a mixture of LAB and *H. alvei* produced the spoilage off-odours typical of spoiling vacuum-packed meats whereas growing the organisms as single cultures did not result in the spoilage off-odours. In cold-smoked salmon, inoculation of three Gram-negative bacteria (*Shewaanella*, *Photobacterium* and *Aeromonas*) did not cause spoilage of vacuum-packed cold-smoked salmon whereas the co-inoculation of the Gram-negative bacteria with *B. thermosphacta* and *Carnobacterium piscicola* produced spoilage off-odours (Joffraud et al., 2001).

In pastuerised milk, both Enterobacteriaceae (*Yersinia intermedia*) and *Pseudomonas putida*. may produce off-odours (Whitfield et al., 2000). However, the amount of esters causing fruity (pine-apple-like) off-odours was increased when the two organisms were co-cultured.

Psychrotolerant *Clostridium* species have been identified as the causative agent of "blown pack" spoilage of vaccum-packed meats (Broda et al., 1996). To our knowledge there has been no reports on influence of interacting microorganisms on this spoilage. However, aerobic bacteria (such as *S. putrefaciens*) significantly affect (enhance) toxin production by *C. botulinum* Type E (Huss et al., 1979) and one may therefore speculate that aerobic bacteria on the meat through their removal of oxygen may create a more advantagous environment for the psychrotrophic spoilage clostridia.

The term "specific spoilage organism" was originally coined to describe the (assumed) single species being responsible for spoilage. Although Jørgensen et al. (2000b) introduced the term "metabiotic spoilage association" to describe situations where two or more microbial species contribute to spoilage through exchange of metabolites or nutrients, this scenario could be covered by the "specific spoilage organisms" concept where it should be specified that a consortium of organisms interact to spoil the product.

4.3. Acylated homoserine lactone-based communication and quroum sensing

The expression of many phenotypic traits in microorganisms is governed by tight gene regulation and influenced by growth phase, nutrients, external stresses and a multitude of other factors. Several behavioural patterns are correlated with the density of the population, and the ability to regulate gene expression as function of cell density has been termed "quorum sensing" (Fuqua et al., 1994). Quorum sensing requires that the microorganisms are capable of communicating by means of chemical signals. Peptides serve as the signal molecules to monitor population size in many Gram-positive bacteria (Kleerebezem et al., 1997). In Gram-negative bacteria, the most intensely studied signals are the *N*-acyl homoserine lactones (AHLs) (Fuqua et al., 1996; Eberl et al., 1999; Whitehead et al., 2001) (Fig. 2). AHL-based quorum sensing systems of the *lux*-homologous family require a minimum of four components for proper function:

(i) a diffusible AHL type signal molecule, synthesised by an enzyme (referred to as the LuxI homolog);
(ii) an AHL-binding receptor (referred to as the LuxR homolog) which DNA binding activity is altered in response to binding of its cognate signal molecule;
(iii) a DNA sequence (typically referred to as the *lux*-box) located in the promoter region of a target gene, acting as target sequence for the regulatory LuxR homolog allowing it to act as transcriptional regulator; and finally
(iv) a set of target genes resulting in a particular phenotype, which is either up-regulated or repressed as a function of the LuxR–AHL binding.

The quorum sensing results from the fact, that AHLs are diffusible over the cell membrane and that the regulatory LuxR–protein requires a threshold concentration of the AHLs in order to be activated. Thus, even though AHLs are produced at low levels in dilute cultures, binding to the receptor protein and the transcriptional activation of target genes only takes place at high cell densities. The binding of the LuxR–AHL complex to the *lux*-box results in transcription of genes downstream of the *lux*-box and this transcrip-

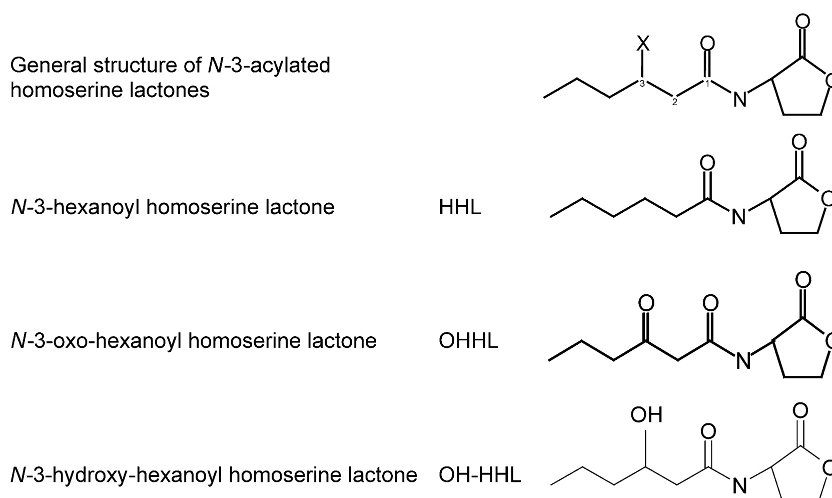

Fig. 2. Structure of typical acylated homoserine lactones. The carbon chain may vary from 4- to 14 carbon-atoms and the third carbon atom may be substituted with an oxo- or a hydroxyl-group.

tion results in an AHL-regulated phenotypic response. In some bacteria, the *luxI*-homologue gene is located downstream of the *lux*-box. Production of the AHL synthase is therefore also regulated by the LuxR–AHL complex, i.e. it is autoinducible. Thus, the activation of the regulatory protein results in a dramatic up-regulation of AHL production and as a consequence AHL regulated phenotypes. As much as a 1000-fold increase in activity per cell has been reported, e.g. for AHL-production and the AHL-regulated bioluminescence in *Vibrio fischeri* (Nealson et al., 1970; Ravn et al., 2001). In other bacteria, AHL-production is constitutive and it appears that the up-regulation of phenotypes is less dramatic (Atkinson et al., 1999; Ravn et al., 2001; Christensen et al., submitted for publication).

The production of AHLs and regulation of different phenotypic traits has been reported in many Gram-negative bacteria (for a recent review, see Whitehead et al., 2001). The quorum sensing system may be advantageous to the bacteria, as they do not waste energy expressing phenotypes (at low cell densities) that are only required at high densities. Classical examples include regulation of symbiotic behaviour (e.g. bioluminescence in *V. fischeri*) or virulence factors (e.g. elastase in *Pseudomonas aeruginosa* (Passador et al., 1993), antibiotic production in *Erwinia carotovora* (Bainton et al., 1992) and Ti-plasmid transfer in *Agrobacterium tumefaciens* (Zhang et al., 1993)) but also more complex behaviour such as surface motility and colonisation of *S. liquefaciens* (Eberl et al., 1996, 1999) and biofilm formation of *P. aeruginosa* (Davies et al., 1998) and *Burkholderia cepacia* (Huber et al., 2001). Cell density dependent regulation of gene expression probably reflects the need for the invading pathogen to reach a critical population density sufficient to overwhelm host defences and thus establish infection. From the area of plant–microbe interactions an elegant example of this was recently published (Mäe et al., 2001). Transgenic plants producing *N*-oxoacyl-homoserine lactone (OHL) showed increased resistance to infecting *E. carotovora*. The plant originating signal molecules force the *E. carotovora* to switch-on production of virulence factors at low bacterial population density. Production of virulence factors elicits a plant defence that due to the low and insufficient bacterial density pushes the balance of the plant–pathogen interaction in the direction of infection abortion.

4.3.1. AHLs in foods and food spoilage bacteria

As out-lined above, the specific spoilage organism concept requires that a link be made by the qualitative and quantitative production of (spoilage) metabolites in an organism, the impact of these metabolites on sensory impression, and the growth of the organism(s) in naturally spoiling products. In particular, the amount of metabolite per cell (e.g. the yield) is an

Table 4
Presence of acylated homoserine lactones in bean sprouts stored at 5 °C (Rosager and Rasmussen, 2001; Gram and Melchiorsen, unpublished data)

Storage time, days	Presence of AHLs by the TraR monitor strain pZLR4	Aerobic plate count, log (cfu/g)
1	+	8.00
6	++	9.30
1	+	7.70
9	++	9.00
1	+	8.95
4	++	9.78

important parameter when evaluating spoilage activity (Dalgaard, 1995). If quorum sensing is used by Gram-negative bacteria involved in spoilage and if AHLs (up)regulate traits relevant to the spoilage behaviour, such information is crucial for evaluation of spoilage activity of an organism. We have set out to determine if AHL-signalling occurs in foods during storage and spoilage, how widespread AHL signalling is amongst Gram-negative spoilage bacteria and to what extend AHL signalling plays a role in bacterial food spoilage.

AHLs can be extracted from complex samples like foods by homogenising in ethyl acetate. Subsequently, the presence of AHLs can be evaluated using one or several bacterial AHL-monitoring strains (McLean et al., 1997; Shaw et al., 1997; Ravn et al., 2001). By using the *Chromobacterium violaceum* CviR (e.g. monitor strain CV026) and the *A. tumefaciens* TraR (e.g. monitor strain with plasmid pZLR4) monitoring systems, we have found that many commercial foods contain large amounts of AHLs (Tables 4–6; Gram et al., 1999). Several types of AHLs are produced in foods and this can be visualised by separation of extracted AHLs on thin layer chromatographic plates and subsequent development by AHL-monitor strains, e.g *A. tumefaciens* pZLR4 (Fig. 3). Also, AHL production in foods can be visualised directly using reporter systems. If an AHL producing strain and an AHL-negative strain carrying an AHL monitor system (e.g. the *luxR*–gene and *luxI*–promoter fused to the *gfp*-reporter gene) are co-inoculated on a piece of meat or salmon, the appearance of green fluorescence in single bacterial cells on the meat/salmon signifies in situ expression of *luxI* and thereby production of AHL (Fig. 4).

Table 5
Presence of AHLs in fish products depending on colony counts and conditions of storage (Rosager and Rasmussen, 2001; Gram et al., 1999)

Food product	Storage temperature (°C)	Storage atmosphere	Storage time (days)	Presence of AHLs		log (cfu/g)	
				LuxR monitor pSB403	TraR monitor pZLR4	Aerobic plate count	Entero-bacteriaceae
Fish fillet	2	plastic bag	0	nd	–	5.30	nd
			10		++	8.30	nd
Fish fillet	2	CO_2-atm.	1	nd	+	3.90	nd
			6		+	5.90	
Fish mince	2	CO_2-atm.	1	nd	+	4.60	nd
			3		+	5.70	
Cold-smoked salmon, 6 samples	5	vacuum	5–8 weeks	–	nd	nr	< 5
Cold-smoked salmon, 4 samples	5	vacuum	5–8 weeks	+	nd	nr	≥ 6
Cold-smoked salmon	5	vacuum	0	nd	–	2.60	nd
			9		–	4.48	
			21		+	7.60	

nd: not done.
nr: not reported.

Table 6
Presence of AHLs in poultry and vacuum-packed beef meat stored at 5 °C (Rosager and Rasmussen, 2001; Bruhn et al., 2002)

Food product	Storage time (days)	Off-odour	pH	Presence of AHLs		Entero-bacteriaceae	log (cfu/g)	
				CviR monitor CVO26	TraR monitor pZLR4		LAB	Aerobic plate count
Vacuum-packed beef, store 1	0	–	5.5	–	–	4.72	5.66	6.53
	4	–		–	–	3.85	6.30	7.08
	7	–		–	–	4.38	7.04	7.76
	14	–		–	+	4.95	6.98	8.49
	21	Sour		–	+	3.57	7.67	8.26
	28	Sour[a]	5.0	–	+	4.74	7.95	8.15
Vacuum-packed beef, store 2	0	–	5.6	–	++	6.56	7.77	7.82
	7	–		–	++	6.58	8.11	8.53
	14	–		–	++	6.63	8.23	8.30
	21	–		–	++	7.11	8.32	8.45
	28	Putrid[a]	5.3	–	++	7.11	8.57	8.78
Vacuum-packed beef, store 3	0	–	5.8	–	++	4.04	6.45	6.45
	7	–		–	+	6.36	8.49	8.56
	14	–		–	++	6.57	8.20	8.71
	21	Putrid		–	+	5.79	7.96	8.34
	28	Putrid[a]	5.6	–	++	6.83	8.36	8.67
Vacuum-packed beef, store 4	0	–	5.3	–	++	6.28	8.20	8.68
	7	–		–	++	6.11	8.26	8.64
	14	–		–	++	6.23	8.26	8.65
	21	Sour		–	++	6.30	8.49	8.54
	28	Sour[a]	5.2	–	++	6.30	8.49	8.62
Vacuum-packed beef, store 5	0	–	5.7	–	++	7.04	7.08	7.89
	7	–		–	++	7.28	7.89	8.74
	14	Putrid		–	++	7.15	8.78	9.00
	21	Putrid[a]	5.5	–	++	7.38	8.48	8.56
Vacuum-packed beef, store 6	2	nd	nd	nd	++	nd	nd	7.60
	23	nd	nd	nd	–	nd	nd	8.11
Turkey, aerobic storage	0	nd	nd	nd	+	nd	nd	8.00
	4	nd	nd	nd	++	nd	nd	8.00
	21	nd	nd	nd	++	nd	nd	9.90

[a] Rejectable based on odour evaluation.

Many Gram-negative bacteria isolated from foods produce AHLs (Table 7), thus all, except one of 148 Enterobacteriaceae isolated from cold-smoked salmon and vacuum-packed meats produced detectable amounts of AHLs (Ravn et al., 2001). AHLs are produced by pseudomonads involved in sprout spoilage (Gram and Melchiorsen, unpublished) and strains of *P. phosphoreum* also produce AHL (Ravn et al., unpublished data). Also, several pectinolytic *Erwinia* and *Pseudomonas*, which spoil ready-to-eat vegetables like bean sprouts, produce AHLs (Rasch et al., in preparation). Thus, AHL production is a widespread phenomenon in food spoiling bacteria. AHL-production can be determined either by random/representative isolation of pure cultures and subsequent determination of AHLs in culture supernatants using the monitors mentioned above. AHL-producing bacteria can also be isolated directly by replica-plating from a non-selective aerobic plates to agar-media containing AHL-monitor strains (Fig. 5).

4.3.2. A potential role for AHLs in food spoilage?

The mere detection of AHLs in foods before and during spoilage and in Gram-negative bacteria that may be involved in spoilage does not allow the

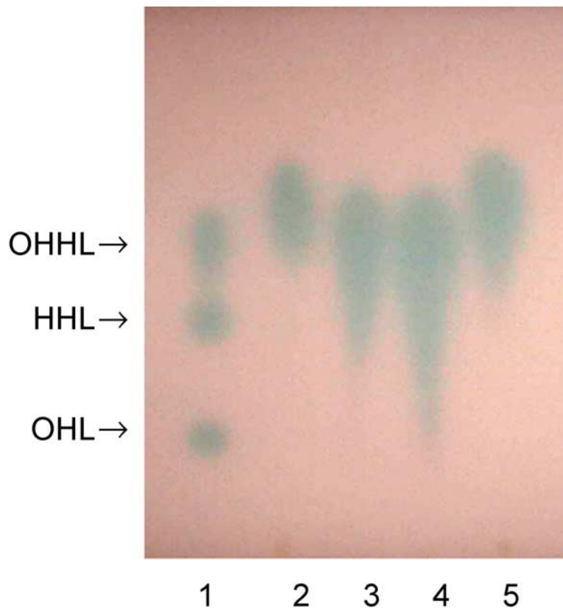

Fig. 3. Thin layer chromatographic separation of AHLs extracted from bean sprouts (lane 2), from pure bacterial cultures isolated from sprouts (lanes 3 and 4) and from sprouts inoculated with AHL producing bacteria (lane 5). Standards of *N*-3-oxo-hexanoyl homoserine lactone (OHHL), *N*-hexanoyl homoserine lactone (HHL) and *N*-octanoyl homoserine lactone (AHLs).

conclusion that the AHL production plays a role in spoilage. To elucidate this, several experimental set-ups can be pursued. Through the construction of signal negative mutants (Christensen et al., submitted for publication), the phenotypes regulated by AHLs can be identified. Further, by comparing the behaviour and sensory impact of AHL-producing wild type strains with non-AHL-producing mutants, possible spoilage reactions regulated by AHL communication can be elucidated.

Storage trials with bean sprouts have demonstrated that inoculation of sprouts with AHL-producing, pectinolytic *E. carotovora* results in a faster spoilage. We are currently studying the potential role of AHLs in these reactions (Rasch et al., in preparation). It is anticipated that the major spoilage reaction is pectin degradation by *Erwinia* spp. and since pectinolytic activity is regulated by AHLs in *E. carotovora* (Pirhonen et al., 1993; Andersson et al., 2000), the hypothesis seems justified.

As mentioned, Enterobacteriaceae isolated from several fish and meat products produce AHLs (Ravn et al., 2001). These organisms may contribute to the spoilage. Leroi et al. (2001) used a step-wise forward multiple regression analysis of data from storage trials with cold-smoked salmon of different origin and found that shelf-life was mostly linked to the initial Enterobacteriaceae count. The higher the initial count on Violet Red Bile Glucose Agar, the shorter the shelf life. Based on biogenic amine production, Jørgensen et al. (2000b) identified *P. phosphoreum* or *L. curvatus* or a mixture of Enterobacteriaceae and lactic acid bacteria as the SSO of cold-smoked vacuum-packed salmon. Also, in vacuum-packed meats, the detection of AHL correlates with the numbers of Enterobacteriaceae being above 10^6 cfu/g (Table 6). At present, it is not known if the ability of these bacteria to produce AHLs plays an important role in meat and fish spoilage.

The spoilage of pasteurised milk is caused by growth of *Bacillus* where spores have survived the heat treatment (Ternström et al., 1993) or by the exo-enzymatic activity of *Pseudomonas* species (re-)contaminating the milk following pasteurisation (Eneroth et al., 2000). It has been suggested that quorum sensing is involved in regulation of hydrolytic activities by milk spoiling pseudomonads (Whan et al., 2000), however, to date (January 2002) no studies have been conducted on this topic. Enterobacteriaceae which produce AHLs may also occur in pastuerised milk (Lindberg et al., 1998; Whitfield et al., 2000). Due to the common involvement of AHLs either directly in regulation of extracellular hydrolytic enzymes (Eberl, 1999) or in-directly through regulation of transport systems (Riedel et al., 2001; Christensen et al., submitted for publication), the hypothesis deserves investigation.

AHLs were readily extracted from both poultry products and vacuum-packed meat products (Table 6). The presence of AHLs in vacuum-packed meats was correlated to the level of Enterobacteriaceae. The involvement of these organisms in spoilage is well documented (Borch et al., 1996), particular in association with LAB (Table 3). It is not yet know if the spoilage reactions of these Enterobacteriaceae are influenced by AHLs.

4.3.3. Other aspects of AHLs in foods

As is evident in Tables 4–6, many of our foods contain AHLs. The major interest in these signalling compounds stems from their role in bacteria–bacteria

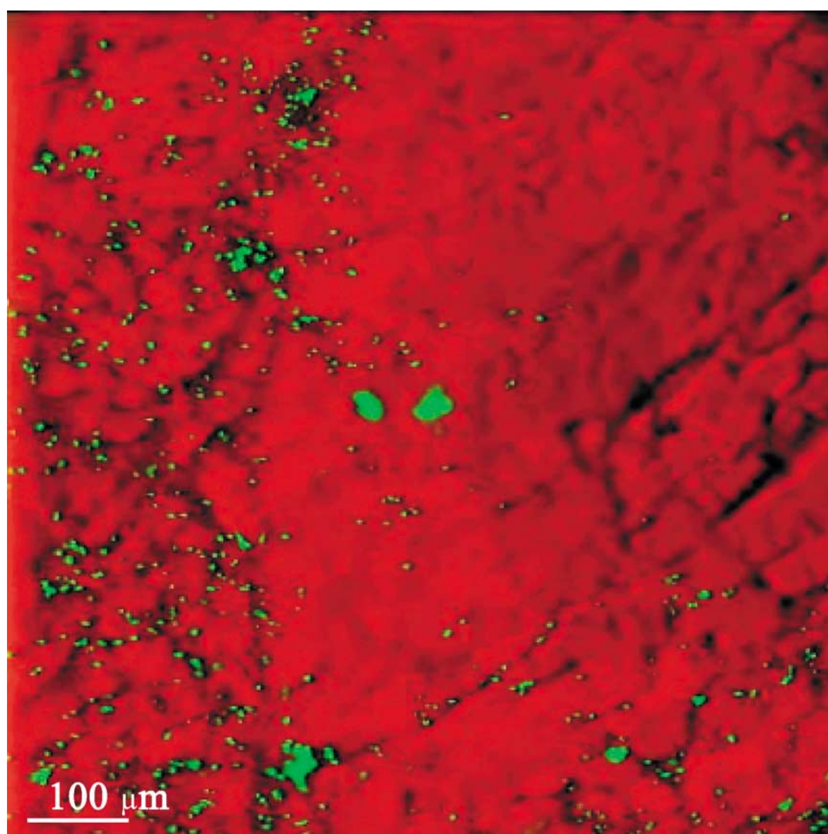

Fig. 4. In situ detection of AHL production in single bacterial cells on meat as assessed by expression of Green Fluorescent Protein. The OHHL producing *H. alvei* was co-inoculated with an OHHL-negative *H. alvei* (*I*-mutant) carrying an AHL-monitor system (*luxR*, *luxI* promoter fused to *gfp*). Green fluorescence was detected using a confocal scanning laser microscope (Christensen, Ravn, Hentzer, unpublished data).

interactions. However, it has recently been demonstrated that AHLs may act directly on eucaryotic organisms and may suppress reactions important for the immune response of epithelial cells (Telford et al., 1998). If AHLs can act directly on gastro-epithelial cells, such action could be a component or mechanism facilitating the attack of food borne pathogens in the gastro-intestinal tract.

To date, little work has been done on stability and degradation of AHLs and it is therefore not known how stable these compounds are in foods and how food preparation procedures affect them. As is shown in Table 6, AHLs detected in vacuum-packed meats may disappear during storage, and Ravn et al. (in preparation) found that degradation of AHLs from food-derived Enterobacteriaceae may be facilitated by the producing organisms. Two recent studies demonstrate that some bacteria are capable of degrading AHLs. Dong et al. (2000) demonstrated that an enzyme from *Bacillus* spp. degraded AHLs and Séveno et al. (2001) suggested that a strain of *Pseudomonas aureofaciens* degrades its own signal molecules.

4.4. Perspectives in food preservation

Due to involvement of AHLs in regulation of virulence factors in several opportunistic human and plant pathogenic bacteria, an intense search has been under way during the last 6 years for compounds that specifically could block AHL-communication. In the clinical scenario, it is envisioned that such compounds can block expression of virulence without affecting growth, thus eliminating the risk of resistance development (Givskov et al., 1996; Finch et al., 1998). Such quorum

Table 7
Production of AHLs by Gram-negative bacteria associated with food spoilage (unpublished data by the authors)

Bacterial group/species	Food commodity	Production of AHL(s)
Enterobacteriaceae	fish	+
	beef	+
	poultry	+
	sprouts	+
Pseudomonas	fish	+
	sprouts	+
Shewanella	fish	− [a]
Photobacterium	fish	+
Aeromonas	fish	+
	poultry	+
	sprouts	+

[a] A few isolates gave a weak response in AHL-monitor strains, which indicates possible production of AHLs, but this has not been further investigated.

sensing inhibitors (QSI) are typically analogues of the AHLs (Eberhard et al., 1986), or compounds that degrade AHLs (Dong et al., 2000). One promising group of QSI is the halogenated furanones produced by the Australian red algae, *Delisea pulchra* (Givskov et al., 1996). These furanones interfere with the receptor proteins and release the AHL signal (Manefield et al., 1999, 2002). Treatment with these compounds reduces expression of AHL-regulated virulence factors and biofilm formation in, e.g. *P. aeruginosa* and *S. liquefaciens* (Hentzer et al., 2002; Rasmussen et al., 2000; Givskov et al., 1996). QSIs like the *D. pulchra* furanones could also be used as food preservatives in selected foods where AHL regulated traits contribute to product spoilage. The finding of QSI compounds from algal (vegetable) sources raises the possibility of identifying active QSI compounds from a multitude of marine organisms as well as from natural foods or food by-products. Recent studies have shown that a number of foods contain furanone compounds with similarity to the *D. pulchra* compounds described above (Slaughter, 1999). QSI compounds may influence colonization of meat surfaces, toxin formation and perhaps bacterial proliferation. The natural occurrence of QSI is an important consideration for assessment of their toxicological status and may facilitate their use in food.

5. Concluding remarks

Many foods spoil due to microbial degradation with their metabolites being the cause of the off-flavours or the textural changes resulting in sensory rejection. Despite the wide range of raw materials, the different processing parameters and the diverse array of storage conditions, very similar microfloras will develop in products with similar physical and chemical characteristics. Only a fraction of the microorganisms present in spoiling food products cause the spoilage characteristics. These organisms are known in some products, but much work is needed on a range of food products to identify the spoilage organisms. Under some condi-

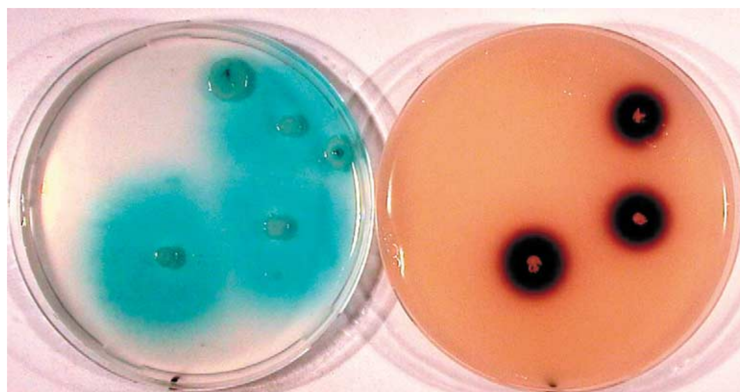

Fig. 5. AHL-producing bacterial colonies visualised by replica-plating from a TSA-plate of a dilution series of vacuum-packed meat onto *A. tumefaciens* pZLR4 in an X-Gal containing medium (left) and onto *C. violaceum* in LB_5-medium (right) (Bruhn et al., in preparation).

tions, also the interactive behaviour of spoilage bacteria may influence their growth and metabolism. Some examples have been described but it is likely that interactive behaviour (metabiosis and antagonism) is important in any foods in which a mixed flora develops during storage. The detection of chemical signals, acylated homoserine lactones, involved in bacterial quorum sensing regulation, in food and food spoiling bacteria is of significant interest. Analyses of the possible importance of AHL compounds in spoilage reactions, and the potential development of novel preservation techniques based on quorum sensing inhibitors, deserves further studies.

Acknowledgements

The authors and the studies on the involvment of AHLs in food quality deterioration was supported by research grants from the Danish Research Council for the Technical Sciences [no. 9700726 and 26000134] and by the Danish Research Academy [PhD grant for L.R.]. We thank Morten Hentzer for assistance with confocal scanning laser microscopy. We extend our thanks to Claus Reesbøll and librarian Søren Tørper Christensen, who helped us get additional references during the revision—for which we were only given 2 weeks.

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