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EFFECT OF DIET SUPPLEMENTATION IN UNSATURATED FATTY ACIDS ON MEAT KEEPING QUALYTIES: STUDY OF SELECTED FATTY ACIDS ANTIMICROBIAL PROPERTIES AND INHIBITION MECHANISM ON *Staphylococcus aureus*

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Esame finale ANNO 2006

I dedicate this work

To my parents Kamdem Jean Marie and Kuissi Jeanne

My brother and sisters Eric, Immaculée, Eliette, Victoire, Glwadys and Fidele

and to my uncle Simo Davide

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Chapter I
INTRODUCTION

Lipids have always been considered as the "good devil" of food products and their presence and composition insure in many of them a characteristic taste before or after cooking. Lipids present in food are generally in the form of phospholipids, triglycerides, diglycerides, monoglycerides and free fatty acids. The structure of these fatty acids also determines the physical, chemical and biological properties of these compounds. From a nutritional point of view, some polyunsaturated fatty acids (PUFA) have gained a lot of interest in the recent years, particularly the omega 3 (ω3) and omega 6 (ω6) PUFA for their positive impact on human health. This is the reason why the presence of this particular class of compounds, mainly the ω 3, is enhanced naturally or by artificial means in some food products. In this scenario, the introduction of vegetable fat in place of animal fat in the diet of livestock animals for the enhancement of the polyunsaturated fraction is becoming a diffused practice. As a consequence of this new approach, the lipid fraction of animal meat has undergone a considerable change in its composition in the animal diet. For adipose tissue and intramuscular fat of swine for example, mathematical relationships between the dietary concentration of polyunsaturated fatty acids and the fatty acid composition of these tissues have been established (Nguyen, et al., 2003). It becomes then important to reconsider the different properties of the lipids fractions of meat products in humans, animal and cells.

I-1 Classification of lipids

On the basis of the physical point of view in particular on their fluidity at room temperature, they are classified as fats or oils, where fats are those mainly composed of saturated fatty acids (FA), while oils are rich of unsaturated ones. Other classifications can be based on the essentiality for humans, on their structure (complex or simple) and on their polarity (neutral or polar lipids). Neutral lipids include fatty acids, alcohols, glycerides and sterols, while polar lipids include glycerophospholipids and glyceroglycolipids. However, this classification can lead to confusion as some short chain fatty acids are very polar, in particular C1 to C3 fatty acids and so it is preferable to use the structure based classification.

Based on their structure, lipids can be classified as derived, simple or complex. The derived lipids include fatty acids and alcohols, which are the building blocks for the simple and complex lipids. Simple lipids, which are composed of fatty acids and alcohol components, include acylglycerols, ether acylglycerols, sterols and their esters and wax esters. In general terms, simple lipids can be hydrolyzed to two different components, usually an alcohol and an acid. Complex lipids include glycerophospholipids (phospholipids), glyceroglycolipids (glycolipids) and sphingolipids. These structures yield three or more different compounds on hydrolysis.

I-1-1 Fatty acids

They are the simplest form of lipids and differ for the length of the carbon chain, the number, position and stereo position of the double bond, the presence of cyclic forms or other functional groups different from the normal carboxylic one.

I-1-1-a Saturated and unsaturated fatty acids

Fatty acids are considered as saturated when the aliphatic chain does not have double bonds as on the contrary it is for the unsaturated ones. Some Authors have proposed to eliminate C1 to C4 carboxylic acids from lipids definition because of their high solubility in water, but the high amount of butyric acid found in dairy fats makes it difficult to apply this. In table 1 and 2 systematic, common and short hand names of the most encountered saturated and unsaturated fatty acids are respectively shown.

Regarding the unsaturated FAs, it can be said that more than 100 mono-unsaturated fatty acids have been described in nature. The most frequent double-bond position is the Δ9, while in some plant it is possible find the presence of Δ5 mono-enes. PUFAs) are best described as families of fatty acids because of the metabolism that allows an interconversion within but not among families of PUFAs (O'keefe, 2002). The essentiality of ω6 FA has been known since the late 1920s, while first evidences of the ω3 importance in human health begun to be accumulated only in the 1970s. Not all PUFAs are essential fatty acids (EFAs). Plants are able to synthesize *de novo* and interconvert ω 3 and ω6 fatty acid families via desaturases with specificity in the Δ12 and Δ15 positions. Animals have Δ5, Δ6 and Δ9 desaturase enzymes and are unable to synthesized the ω3 and ω6 PUFAs de novo. However, extensive elongation and de- saturation of EFA occurs (primarily in the liver). The elongation and desaturation of 18:2 ω6 is illustrated in Figure 1. 18:2 ω6 is the most common ω6 fatty acid in our diets . Often considered the parent of the ω6 family, 18:2 ω6 is first desaturated to 18:3 ω6. The rate of this first desaturation is thought to be limiting in premature

infants, in the elderly, and under certain disease states. Thus, a great deal of interest has been placed in the few oils that contain 18:3 ω6, γ-linolenic acid (GLA). Relatively rich sources of GLA include black currant, evening primrose, and borage oils. GLA is elongated to 20:3 ω6, dihomo- γ -linolenic acid (DHGLA). DHGLA is the precursor molecule to the 1-series prostaglandins. DHGLA is furtherly desaturated to 20:4 ω6, precursor to the 2-series prostaglandins. A further elongation and desaturation to 22:4 ω6 and 22:5 ω6 can occur, although the exact function of these fatty acids remains obscure (O'keefe, 2002).

Table1: Systematic, common and short hand names of saturated FAs.

Systematic name	Common name	Shorthand
Methanoic	Formic	1:0
Ethanoic	Acetic	2:0
Propanoic	Propionic	3:0
Butanoic	Butyric	4:0
Pentanoic	Valeric	5:0
Hexanoic	Caproic	6:0
Heptanoic	Enanthic	7:0
Octanoic	Caprylic	8:0
Nonanoic	Pelargonic	9:0
Decanoic	Capric	10:0
Undecanoic	—	11:0
Dodecanoic	Lauric	12:0
Tridecanoic	—	13:0
Tetradecanoic	Myristic	14:0
Pentadecanoic	—	15:0
Hexadecanoic	Palmitic	16:0
Heptadecanoic	Margaric	17:0
Octadecanoic	Stearic	18:0
Nonadecanoic	—	19:0
Eicosanoic	Arachidic	20:0
Docosanoic	Behenic	22:0
Tetracosanoic	Lignoceric	24:0
Hexacosanoic	Cerotic	26:0
Octacosanoic	Montanic	28:0
Tricontanoic	Melissic	30:0
Dotriacontanoic	Lacceroic	32:0

Figure 2 illustrates analogous elongation and desaturation of 18:3 ω3. The elongation of 20:5 ω3 to 22:5 ω3 has been thought for many years to be via Δ4 desaturase. The inexplicable difficulty in identifying and isolating the putative Δ4 desaturase led to the conclusion that it did not exist, and the

pathway from 20:5 ω3 to 22:6 ω3 was elucidated as a double elongation, desaturation, and β-oxidation.

One of the main functions of the EFAs is their conversion to metabolically active prostaglandins and leukotrienes (Granstrom and Kumlin,1987; Slater and McDonald-Gibson 1987). Examples of some of the possible conversions from 20:4 ω6 are shown in Figures 3 and 4 (Slater and McDonald-Gibson 1987). The prostaglandins are called eicosanoids as a class and originate from the action of cyclooxygenase on 20:4 ω6 to produce PGG_2 . The standard nomenclature of prostaglandins allows usage of the names presented in Figure 3. For a name such as PGG_2 , the PG represents prostaglandin, the next letter (G) refers to its structure, and the subscript number refers to the number of double bonds in the molecule.

Table 2: Systematic, common and short hand names of unsaturated FAs.

Systematic name	Common name	Shorthand
c-9-Dodecenoic	Lauroleic	12:1ω3
c-5-Tetradecenoic	Physeteric	14:1ω9
c-9-Tetradecenoic	Myristoleic	14:1ω5
c-9-Hexadecenoic	Palmitoleic	16:1ω7
c-7,*c*-10,*c*-13-Hexadecatrienoic	—	16:3ω3
c-4,*c*-7,*c*-10,*c*-13-Hexadecatetraenoic	—	16:4ω3
c-9-Octadecenoic	Oleic	18:1ω9
c-11-Octadecenoic	*cis*-Vaccenic (Asclepic)	18:1ω7
t-11-Octadecenoic	Vaccenic	[a]
t-9-Octadecenoic	Elaidic	[a]
c-9,*c*-12-Octadecadienoic	Linoleic	18:2ω6
c-9-*t*-11-Octadecadienoic acid	Ruminic[b]	[a]
c-9,*c*-12,*c*-15-Octadecatrienoic	Linolenic	18:3ω3
c-6,*c*-9,*c*-12-Octadecatrienoic	γ-Linolenic	18:3ω6
c-6,*c*-9,*c*-12,*c*-15-Octadecatetraenoic	Stearidonic	18:4ω3
c-11-Eicosenoic	Gondoic	20:1ω9
c-9-Eicosenoic	Gadoleic	20:1ω11
c-8,*c*-11,*c*-14-Eicosatrienoic	Dihomo-γ-linolenic	20:3ω6
c-5,*c*-8,*c*-11-Eicosatrienoic	Mead's	20:3ω9
c-5,*c*-8,*c*-11,*c*-14-Eicosatrienoic	Arachidonic	20:4ω6
c-5,*c*-8,*c*-11,*c*-14,*c*-17-Eicosapentaenoic	Eicosapentaenoic (EPA)	20:5ω3
c-13-Docosenoic	Erucic	22:1ω9
c-11-Docosenoic	Cetoleic	22:1ω11
c-7,*c*-10,*c*-13,*c*-16,*c*-19-Docosapentaenoic	DPA	22:5ω3
c-4,*c*-7,*c*-10,*c*-13,*c*-16,*c*-19-Docosahexaenoic	DHA	22:6ω3
c-15-Tetracosenoic	Nervonic (Selacholeic)	24:1ω9

[a]Shorthand nomenclature cannot be used to name *trans* fatty acids.
[b]One of the conjugated linoleic acid (CLA) isomers.

Figure 1: Pathway of 18:2ω6 metabolism to 20:4 ω

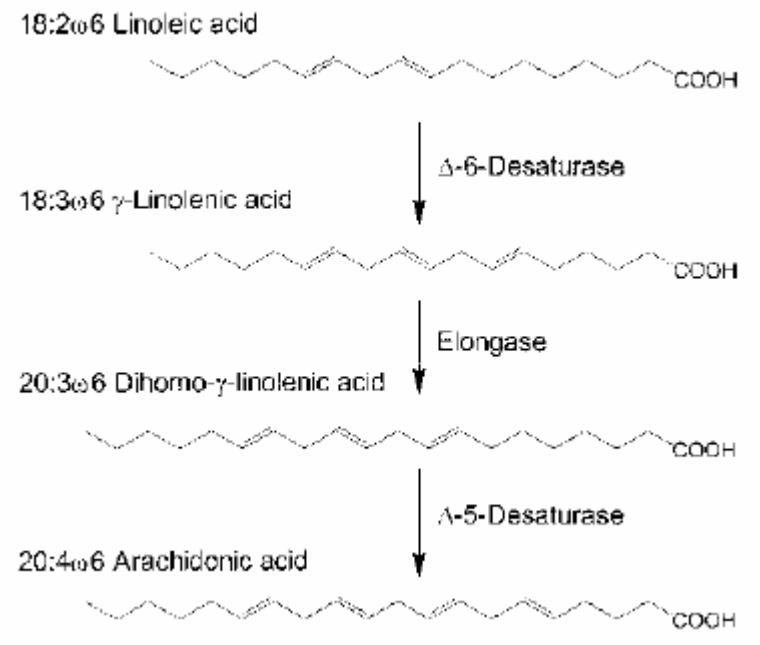


Figure 2: Pathway of 18:ω3 metabolism to 22:6 ω3.

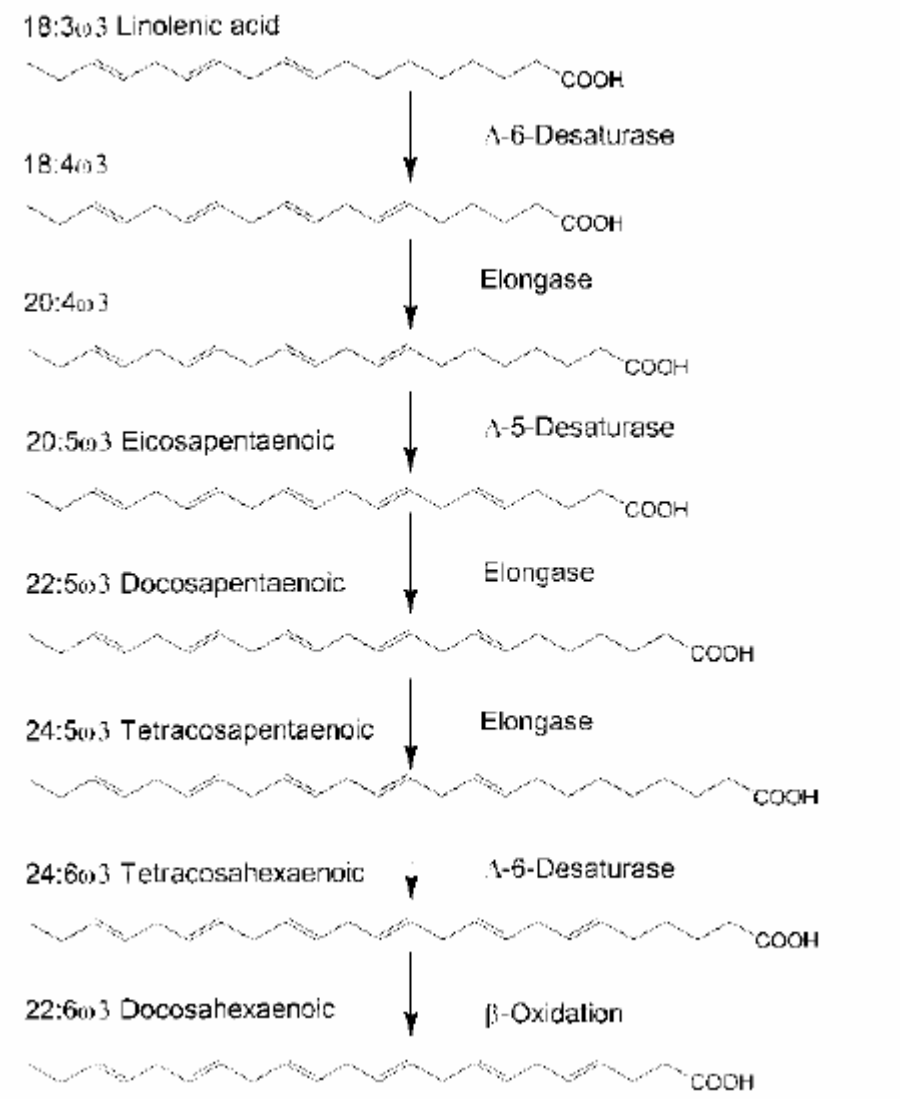


Figure 3: Prostaglandin metabolites of 20:4ω6.

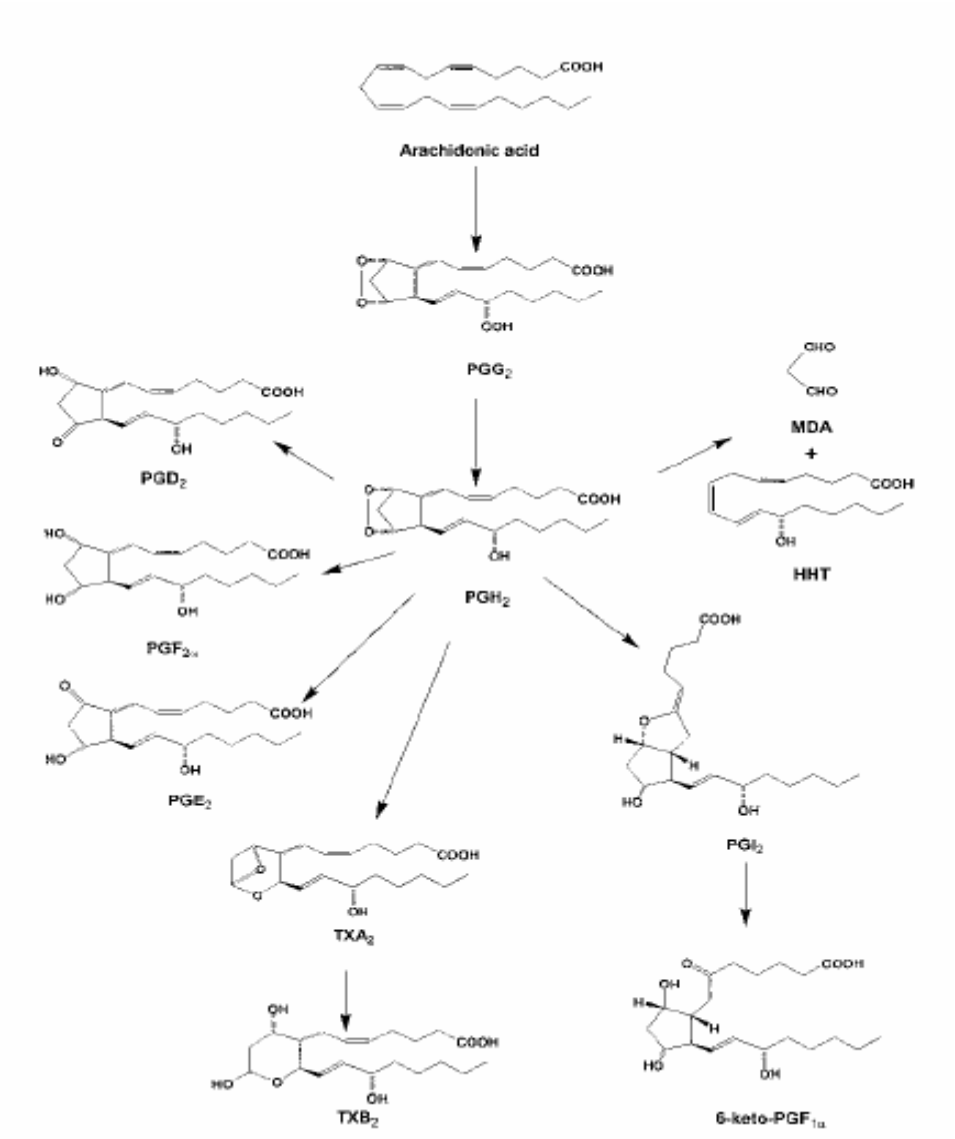
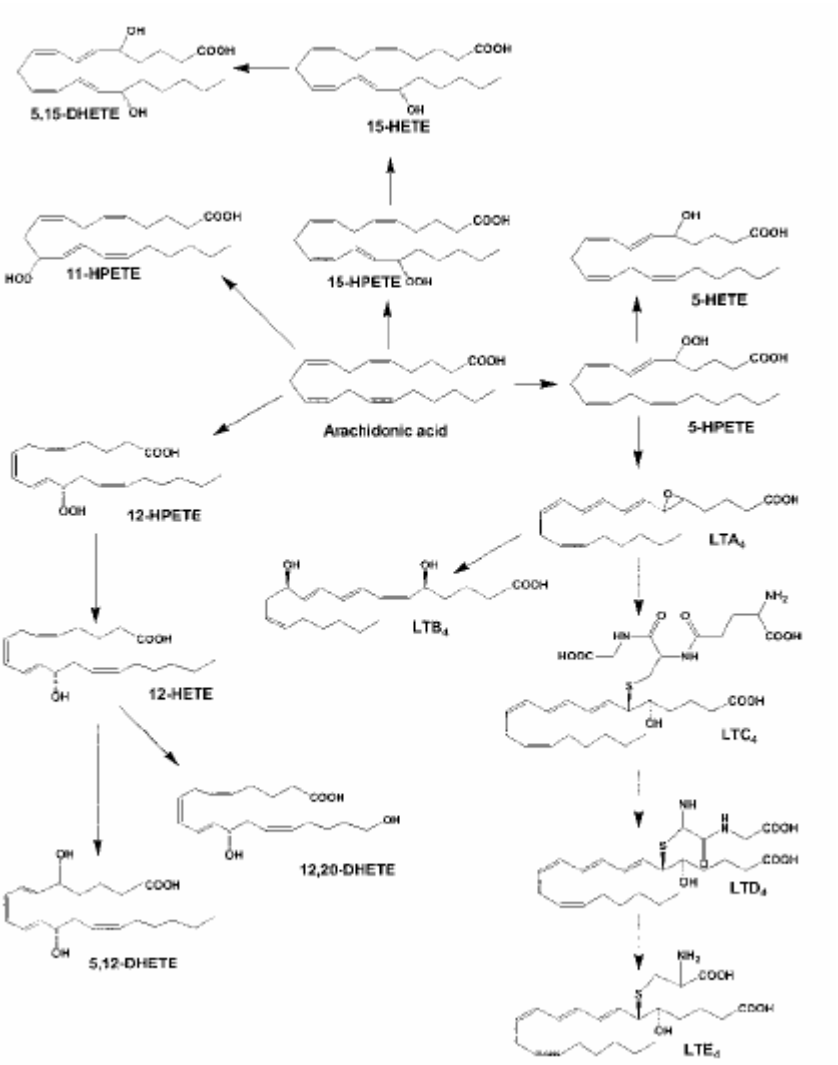


Figure 4: Leucotriene metabolites of 20:4ω6.



I-1-1-b Acetylenic fatty acids

They are fatty acids with triple bonds, generally found with unsaturation beginning from Δ9 and in most of the cases with 18 carbons. They are very rare and some examples are stearolic acid (9-octadecynoic acid) and ximenynic acid (trans-11-octadecene-9-ynoic acid).

I-1-1-c Trans fatty acids

Trans fatty acids are not frequent in nature, and their presence in food is due to three principal factors: bacterial metabolism, deodorization of oils and partial hydrogenation of oils. The configuration of isolated double bonds in naturally occurring lipids of eukaryotes is *cis*. However, *cis–trans* isomerization of lipid double bonds occurs in some bacteria enzymatically (Keweloh and Heipieper,1996; Heipieper,et al., 2003) and *trans* isomers are present in mammalian cells after dietary supplementation of chemically modified fats (Se´be´dio, 1998). Some trans geometrical isomers found in living organisms can only arise via an endogenous transformation of the naturally occurring cis structures and are correlated with radical stress produced during physiological and pathological processes (Zghibeh et al., 2004 and Ferreri et al., 2005).

I-1-1-d Branched fatty acids

The most common branched fatty acids are the *iso* and *anteiso* FAs. When the methyl branch is on the penultimate (ω2) carbon, the compound is said to be *iso*, while if it branches on the third-ultimate (ω3) carbon, it is *anteiso*. The *iso* and *anteiso* fatty acids are thought to originate from a modification of the normal *de novo* biosynthesis, with acetate replaced by 2-methyl propanoate or 2-methylbutanoate, respectively Gunstone, Harwood.and Padley (1994). Other branched fatty acids are derived from isoprenoid biosynthesis including pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and phytanic acid (3,7,11,15 tetramethylhexadecanoic acid). In bacteria, both *iso* and *anteiso*-branching are created by *de novo* synthesis, and the position of the branch cannot be changed post-synthesis. The branching positions are determined by the type of amino acid (leucine for iso-branching and isoleucine for anteiso-branching) used by the fatty acid synthetase or AcylCoA:ACP transacylase (Russell, 1984; 1995; 1997).

I-1-1-e Cyclic fatty acids

Many fatty acids that exist in nature contain cyclic carbon rings (Sébédio and Grandgirard, 1989). Ring structures contain either three (cyclopropyl and cyclopropenyl), five (cyclopentenyl), or six (cyclohexenyl) carbon atoms and may be saturated or unsaturated. As well, cyclic fatty acid structures resulting from heating the vegetable oils have been identified (Sébédio and Grandgirard,

1989; LeQuere et al., 1991). Cyclopropane fatty acids are commonly found in Gram-negative and on rare occasions also in Gram-positive bacteria (Russell, 1984). Cyclopropane fatty acyl chains confer fluidity upon the cell membrane and assist in tolerance towards disturbance (Eze and McElhaney, 1981; Russell, 1984). An example of cyclopropyl fatty acids is the lactobacillic acid also called phytomonic acid whose IUPAC name is 10-(2-hexylcyclopropanyl) decanoic acid. However, the amount of cyclopropane fatty acyl chains in the membrane does not change significantly in response to disturbance (Russell, 1984; Diefenbach et al., 1992). Cyclopropane fatty acids are predominantly produced by a cyclase enzyme, but can also be produced by post-synthesis modification of *cis*-unsaturated fatty acids (Russell, 1984; Diefenbach et al., 1992).

I-1-1-f Hydroxy and epoxy fatty acids

Cutins, which are found in the outer layer of fruit skins, are composed of hydroxy acid polymers, which may contain also epoxy groups (Gunstone, Harwood, and Padley. 1994). Epoxy acids, found in some seed oils, are formed on prolonged storage of seeds (Gunstone et al., 1994). Plant systems produce hydroxy fatty acids, which are important industrial materials. Hydroxy fatty acids are plant self-defense substances (Masui et al, Phytochemistry 1989). They are used in plasticizers, surfactants, lubricants, and additives and in the manufacture of paints because the hydroxy group gives materials special properties, such as higher viscosity and reactivity to fatty acids (Hou, 2000). Furthermore, some hydroxy fatty acids are known to have interesting biological activities. For example, Kato et al. (1984) and other researchers (Bowers,et al., 1986; Hou and Forman III, 2000; Masui, et al., 1989) reported that hydroxy fatty acids have antifungal activity. Sjögren, et al., (2003) reported the identification and chemical characterization of four antifungal substances, 3-(*R*)-hydroxydecanoic acid, 3-hydroxy-5-*cis*-dodecenoic acid, 3-(*R*)-hydroxydodecanoic acid and 3-(*R*)-hydroxytetradecanoic acid, from *Lactobacillus plantarum.* A few hydroxy fatty acids also exhibit cytotoxic activity against cancer cells (Kawagishi, et al., 1994; Stadler, et al., 1994) and prostaglandin E-like activity (Wang and Wang, 1996). Recently, production of hydroxy fatty acids through bioconversion by microorganisms has been a major focus of research (Hou, 1995). A number of microbial systems that convert oleic acid to monohydroxy and dihydroxy fatty acids have been found (Andres, e al., 1994; Blank, et al., 1991; El-Sharkawy et al., 1992; Hou and Baghy, 1991; Hou et al., 1991; Wallen et al., 1962). Bioconversion of polyunsaturated fatty acids, such as linoleic acid and α- and γ-linolenic acids, has also been studied (Hou, 1995; Koritala and Bagby., 1992; Wallen et al., 1971; Weil et al., 2002). Lactic acid bacteria (LAB) have a long history of use as biopreservatives for food and feed storage. The general

preserving ability of lactic acid and other fermentation end products and the antibacterial effects of LAB proteinaceous bacteriocins are well documented (Lindgren and Dobrogosz. 1990; Stiles 1996). Recent research has revealed that LAB can produce low-molecular-weight antifungal substances, e.g., phenyllactic acid, *p*-hydroxyphenyllactic acid (Lavermicocca, et al., 2000; Stro¨m et al., 2002), cyclic dipeptides such as cyclo(Gly-L-Leu), cyclo(L-Phe-L-Pro), and cyclo(L-Phe-*trans*-4-OH-L-Pro) (Niku-Paavola, et al., 1999; Ström et al., 2002), benzoic acid, methylhydantoin, mevalonolactone (Niku-Paavola, et al., 1999), and short-chain fatty acids (FAs) (Corsetti, et al., 1998).

I-1-1-g Furanoid fatty acids

They are fatty acids that contain an unsaturated oxolane heterocyclic group. The are commonly called furanoid fatty acids because of the furan structure that constitutes the base of the molecule structure. They are majors components of latex rubber (Christie, 1982; Gunstone et al., 1994), but are also found in plants, algae, bacteria, marine oil, liver and testes.

I-1-2 Acylglycerols

These compounds are the predominant constituent in oils and fats of commercial importance. When the glycerol is esterified with one, two or three fatty acids in one or all the hydroxyl positions, the result is the formation of monoacyl, diacyl and triacylglycerol, respectively. The major part of free fatty acids found in food products are generally produced by hydrolysis of acylglycerols.

I-1-3 Sterols and sterol esters

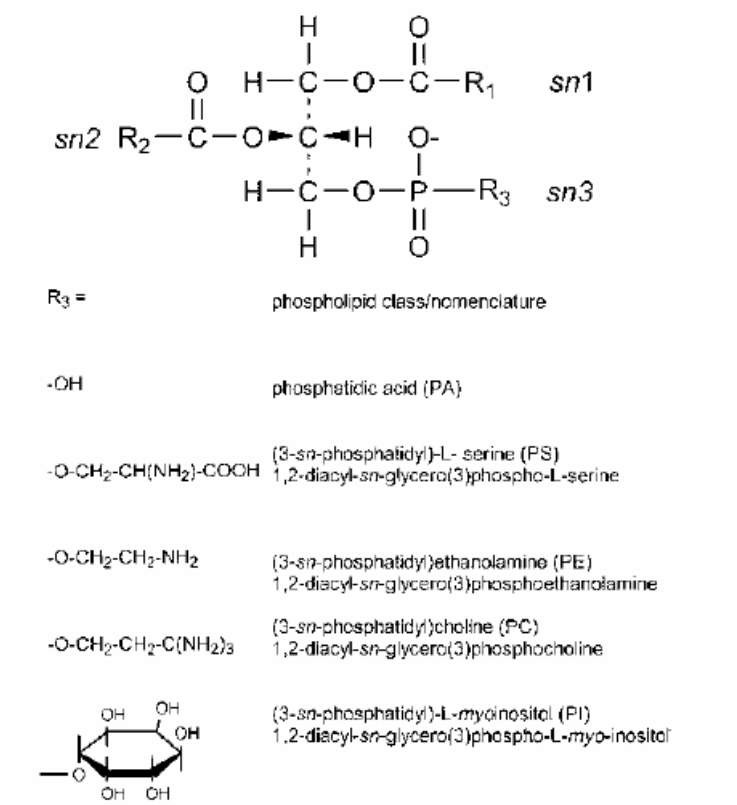
The steroid class of organic compounds includes sterols of importance in lipid chemistry. Although the term ‘‘sterol’’ is widely used, it has never been formally defined. The following working definition has been proposed some years ago: ‘‘Any hydroxylated steroid that retains some or all of the carbon atoms of squalene in its side chain and partitions nearly completely into the ether layer when it is shaken with equal volumes of ether and water (Nes and McKean, 1977). Thus, according to this definition, sterols are a subset of steroids and exclude the steroid hormones and bile acids. The importance of bile acids and their intimate origin from cholesterol makes this definition difficult. As well, non-hydroxylated structures such as cholestane, which retain the steroid structure, are commonly considered sterols. The sterols may be derived from plant (phytosterols) or animal (zoosterols) sources. They are widely distributed and are important in cell membranes. The predominant zoosterol is cholesterol. Although a few phytosterols predominate, the sterol

composition of plants can be very complex. For example, as many as 65 different sterols have been identified in corn (*Zea mays*) (Guo, Venkatramesh and Nes 1995).

I-1-4 Phospholipids

Phosphoglycerides (PLs) are composed of glycerol, fatty acids, phosphate and (usually) an organic base or polyhydroxy compound. The phosphate is almost always linked to the sn-3 position of the glycerol molecule. The parent structure of the phosphoglycerides is the phosphatidic acid (*sn*-1,2-diacylglycerol-3-phosphate). The terminology for phosphoglycerides is analogous to that of acylglycerols with the exception of the no acyl group at *sn*-3. The prefix lyso-, when used for phosphoglycerides, indicates that the *sn*-2 position has been hydrolyzed and a fatty acid is esterified to the sn-1 position only. Some common phosphoglyceride structures and nomenclature are presented in Figure 5. Phospholipid classes are denoted using shorthand designation (PC = phosphatidylcholine, etc.). The standard nomenclature is based on the PL type. For example, a PC with an oleic acid on sn-1 and linolenic acid on *sn*-2 would be named 1-oleoyl-2-linolenoyl-sn-glycerol-3-phosphocholine.

Figure 5: Some of the .most common phospholipids



I-1-5 Other lipid classes

Other lipid classes are :

- **Waxes**: also called wax ester, they are esters of fatty acids with long chain alcohols. Simple waxes are found on the surface of animals, plants and insects and play a role in the prevention of water loss.
- **Ether(phosphor)glycerides (plasmalogens)**: they are formed when a vinyl (1-alkenyl) ether bon is found in a phospholipids or acylglycerol.
- **Glyceroglycolipids** : also named glycolipids, they are formed when a 1,2-diacyl-*sn*-3-glycerolis is linked via the *sn*-3 position to a carbohydrate molecule. Galactose is the most common carbohydrate in the plant glyceroglycolipids.
- **Sphingolipids**: The glycosphingolipids are a class of lipids containing a long chain base, fatty acids, and various other compounds, such as phosphate and monosaccharides. The base is commonly sphingosine, although more than 50 bases have been identified. The ceramides are composed of sphingosine and a fatty acid.

I-2 The role of lipids

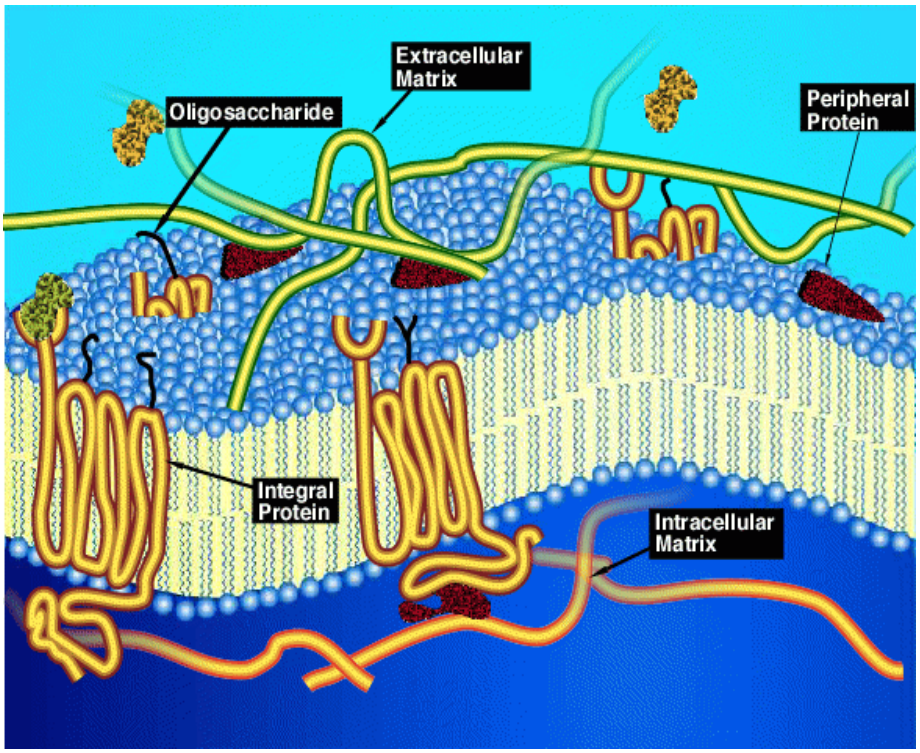
I-2-1 Role of lipids in the structure and function of biological membranes

What is common to all biological membranes is the presence in relatively large amount of some amphipatic molecules, that can be sulfolipid, phospholipid or glycolipid. In the membranes of animal cells, phospholipids are the predominant species of amphipathic molecules; in the membranes of plant cells, sulfolipids and glycolipids are on a par with phospholipids as far as relative amounts of each are concerned. There is great variability not only in respect to the nature of the polar sector of the amphipathic molecule, but also in respect to the hydrocarbon or nonpolar sector. The latter sector may contain a fatty acyl, fatty aldehydic, fatty alcoholic, or even an isoprenyl residue. The residues in the hydrocarbon sector must contain a sufficient number of carbon atoms in a linear chain to reduce the solubility of the bimodal molecule in water to negligible amounts. The critical number of carbon atoms per residue appears to be about 18 (Green and Tzagoloff, 1966). The fatty residues of the phospholipids of animal and plant mitochondria are generally polyunsaturated, whereas the phospholipids of bacterial membranes are generally monounsaturated. The fatty residues in each membrane appear to have a distinctive pattern of unsaturation; this pattern may vary widely from one membrane to another one within the same cell.

Lipids are hence a very important part in the structure of biological membranes. They are the fundaments of the membrane fluidity and adaptation capacity in different environments. In bacteria, the lipid bilayer forms the framework of the cytoplasmic membrane. The primary lipid components of this bilayer are the polar glycerophospholipids although other polar lipids such as sphingophospholipids, sphingo and glycerogylcolipids as well as neutral lipids (hopanoids and carotenoids) may contribute to the structure of the membrane (Ratledge and Wilkinson, 1988). Ions (i.e. Na+, K+), water, proteins and carbohydrates are also found within the lipid bilayer. The most widely accepted structure of the membrane is a modified version of the Fluid Mosaic Model by Singer and Nicolson (1972). This model envisions proteins as globular entities embedded within the fluid lipid bilayer (figure 6). Proteins may be located at the periphery (peripheral proteins), span the membrane in part (integral proteins) or completely transverse the membrane (transmembrane proteins). Carbohydrate portions can be found attached to proteins or lipids and extend outwards from the membrane (Finean et al., 1978).

Bacterial cytoplasmic membranes are both functionally and structurally diverse. Cytoplasmic membranes define cells from the external environment, contain the cytoplasm and other cellular constituents. They regulate the movement of substances entering or exiting cells and catalyze exchange reactions. Membranes also play a role in energy transduction and in the maintenance of ion and solute gradients to maintain a constant intracellular environment. They also provide a milieu where biological reactions can occur and act to regulate cellular growth and metabolism. Cytoplasmic membranes stabilize protein structure, which is important with regard to the function of membrane embedded enzymes. In addition, membrane embedded molecules and receptors add to the role of the membrane by allowing it to provide for intercellular communication and detection of cellular signals (Denich, Beaudette, Lee and Trevors, 2003).

Figure 6: Representation of the fluid mosaic model of the cytoplasmic membrane.



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I-2-2 Importance of lipids in human diet

Most discussions on fats and health focus on the deleterious effects of these essential nutrients. What it is discussed in that case is the possibly harmful effects of an excess of fats and oils. Fats (lipids) supply energy, support structural aspects of the body and provide substances that regulate physiological processes.

Adipose tissue, which is the repository of most of our body fats, serves as an energy reservoir (fat supplies nine calories per gram compared to four calories per gram for protein or carbohydrate), as a heat conserver and as a shock absorber. Lipids contain essential fatty acids, such as linoleic and linolenic acids. These are metabolized eventually to provide eicosanoids, substances that possess hormone-like activity and thus may regulate many body functions. Fat is also the transport vehicle for vitamins A, D, E, and K.

Polyunsaturated ω 3 FA can for example exert immune modulating and organ protective effects (Heller et al., 1998; Heller et al., 2003) Depending on nutritional intake, ω 3 FA are incorporated in the phospholipid pool of cellular membranes and replace the ω 6 FA thereby increasing membrane fluidity and influencing lipid mediator and cytokine production (Breil et al., 1996; Mayer et al., 2003). The concept that nutritional supplementation with ω 3 FA exerts immunomodulatory and organ protective effects that are based on the multiple interactions of ω 3 FA is summarized in Figure. 7.

Figure 7: Effects of ω6/ ω3 FA ratio on the regulation of inflammation by (Koch and Heller 2005)

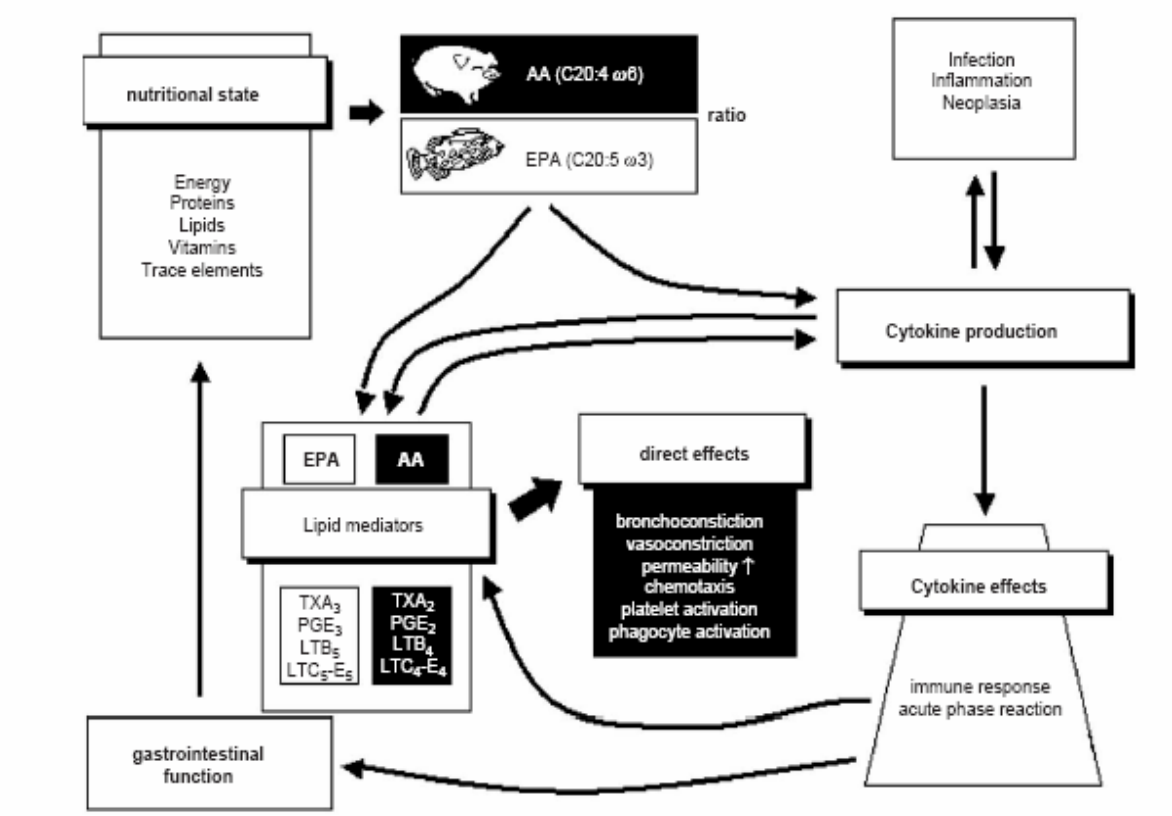


Figure 7:The human nutritional condition is determined by the constituents of nutrition. Concerning lipids, the ratio between ω 3 and ω 6 FA determines the type and intensity of the inflammatory reaction, in terms of lipid mediators and cytokine production. While arachidonic acid (AA)-metabolites (black boxes) may induce hyperinflammation, eicosapentaenoic acid (EPA)-derived mediators are more immunoneutral. Cytokine production itself as part of the acute phase response is closely coupled to lipid mediator generation via positive feed back loops, for example, by triggering the expression of COX II or iNOS. These complex interactions apply for infection and inflammation to the same degree as they do for tumor cell defense. As a result of over activated immune responses, gastrointestinal function may be impaired and

Cholesterol, which has absorbed the brunt of the anti-fat attack, is a compound that is essential for life. It is not essential in the sense of essential fatty acids since the body can synthesize it, but it is a crucially important component of our biological economy. Cholesterol comprises about 0.2% of normal body weight. Most of it (about 33%) is in the brain and nervous system where its function has not been probed beyond suggesting that its major function is as an insulator. Almost another one-third of the body's cholesterol is in muscle where it is a structural component. Every cell membrane contains cholesterol and phospholipid, another fatty substance. The esterified cholesterol found in muscle may represent a storage compartment. The percentage of cholesterol ester in muscle increases with age. Cholesterol is the parent substance for vitamin D2, bile acids, adrenocortical hormones, and sex hormones. Thus, it is one of the most important biological substances. Fat also contributes to the palatability and flavour of food and hence to the enjoyment of eating.

The two major causes of death in the developed world are heart disease and cancer. Both have been described as lifestyle diseases, and effects of diet fit under that rubric. Since diet is one of the easiest lifestyle factors to investigate and possibly change, its role has been pursued with vigour. However, dietary data are not as simple to obtain as one might expect. Population-based data, derived from availability statistics, do not account for individual variations. Recall may be flawed by habitual underreporting of intake (Mertz et al., 1991; Mertz, 1992). These methods provide useful data, but their shortcomings should be kept in mind (Kritchevsky, 2002).

I-2-2-a Lipids and coronary heart disease

In this research area it is difficult to conduct experiment series with univocal factors excluding others. But the large quantities of data collected over decades have brought to interesting associations between excess fat consumption and coronary heart diseases (CHD). The major risk factors for CHD, include cigarette smoking, elevated cholesterol level, elevated blood pressure, obesity, and maleness (Kritchevsky, 2002). There is a wealth of epidemiological and experimental evidence indicating that a diet high in saturated fatty acids (SFA) is associated with a high level of serum cholesterol which in turn is related to high incidences of CHD, although without a direct correlation. The raised level of cholesterol, especially of low density lipoprotein cholesterol (LDL), is taken up to macrophages and deposited in the plaque (Ulbright and Southgate, 1991). But not all SFA are equally likely to cause hypercholesterolemia; stearic acids do not raise serum cholesterol (Bornanome and Grundy1988) as well as short-chain SFA (C10 and shorter ones). Hence the

putative atherogenic SFA are C12:0, C14:0 and C16:0, a fact already recognised by Keys (1965). Based on these facts, the commonly used PUFA/SFA ratio as indicator or measurement of the level of atheogenicity or promotion of CHD is obviously inappropriate, since only three of the SFA are hypercholesterolaemic (Ulbright and Southgate, 1991). Regarding thrombus formation, it is generally accepted that long-chain fatty acids accelerate their production, while PUFA and monounsaturated FA (MUFA) do not (McGregor et al., 1980). But butter-enriched (but not lard-enriched) diets reduce the production of arterial prostacylin, a prostaglandin that is strongly antagonist of pallet aggregation (O'Dea et al., 1988).

ω6 PUFA, whose parent member is the linoleic acid, are beneficial in protecting against atheroma only if moderately increased in the diet (Ulbright and Southgate, 1991), because their high quantity in diet can lead at the same time to high-density lipoprotein depression and low density lipoprotein lowering. So the ability of fatty acids to inhibit platelet aggregation is inversely correlated to their melting point (hence SFA<MUFA<PUFA in inhibitory activity). This effect of *cis*-PUFA on platelet activation is therefore related to an increase in the platelet membrane fluidity. The high ratio of ω6 to ω3 PUFA increase the risk of thrombosis, while *trans*-PUFA, in contrast to *cis*-PUFA are said to increase (rather than inhibit) the aggregation of platelets by thrombin (Ulbright and Southgate, 1991).

Regarding ω3 PUFA, alpha-linolenic acid (C18:3) is the most found in the green tissue plants, while in animals it is converted to a series of longer-chain PUFA of which the most important are eicosapentaenoic acid (EPA- C20:5) and docosahexaenoic acid (DHA- C22:6). These two fatty acids are particularly effective in inhibiting the platelets aggregation and act by inhibiting the arachidonic acid convertion into platelets aggregating thomboxane (thomboxanes A2) (Ulbright and Southgate, 1991). It has then been concluded by Renaud et al. (1986) that the clotting activity of platelets and their aggregation to thrombus is closely related to the intake of long-chain SFA and that the principal effect of increasing the intake of ω6 PUFA is antiatherogenic (reduction of serum lipids), whereas that of ω3 PUFA is antithrombogenic (reduction of platelet activity).

On the base of the effective involvement of fatty acids in atherogenicity and thrombogenicity, Ulbright and Southgate (1991) proposed two new formula for the calculation of an index of atherogenicity (IA) and index of thrombogenicity (IT) in food.

$$IA = \frac{aS' + bS'' + cS'''}{dP + eM + fM'}$$

Where *S'*=C12:0, *S''*= C14:0 and *S'''*=C16:0; P= sum of ω6 PUFA and ω3 PUFA; M= oleic acid (C18:1); and M'= sum of other MUFA; a-f are empirical constants based on the incidence of the different groups on atherogenicity; b has been set to 4, while a, c, d , e and f have been provisionally set as unit.

$$IT = \frac{mS^{iv}}{nM + oM' + p(\omega 6) + q(\omega 3) + \frac{\omega 3}{\omega 6}}$$

Where S^{iv}=sum of C14:0, C16:0 and C18:0; ω6= ω6 PUFA; ω3= ω3 PUFA, M an M' as before; m,n,o,p and q are unknown constant with the following up to knowledge assign values; m has been set as unity, while n, o and p have the value 0.5 because MUFA and ω6 PUFA are less antiatherogenic than ω3 PUFA; q has the value 3.

Table 3: Indices of atherogenicity and thrombogenicity of some foods and diets. Ulbright and Southgate, (1991)

Food and diets	Index of atherogenicity	Index of thrombogenicity
Coconut oil	13.63	6.18
Milk, butter, cheese	2.03	2.07
Palm oil	0.88	1.74
Lamb:		
Roast breast, lean & fat	1.00	1.58
Chop, lean only	1.00	1.33
Beef:		
Topside roast, lean	0.72	1.06
Raw mince	0.72	1.27
Grilled sausage	0.74	1.39
Pork:		
Roast leg, lean	0.60	1.37
Grilled sausages	0.58	1.35
Fried steaky bacon, lean & fat	0.69	1.66
Hard margarine (veg oils only)	0.56	1.26
Stewed ox liver	0.41	0.82
Chicken, roast, meat & skin	0.50	0.95
PUFA margarine	0.35	0.53
Olive oil	0.35	0.53
Sunflower oil	0.07	0.28
Raw mackerel	0.28	0.16
Eskimo diet	0.39	0.28
Danish diet	1.29	1.51
British diet	0.93	1.21

I-2-2-b Lipids and obesity

Obesity is in general defined as a situation of body weight of more than 20% higher than the ideal body weight or body index [weight (Kg)/ height (cm)] (Whitney and Rolfes, 1993). It is becoming a huge problem in the industrialized countries as more than one third of the population is suffering of obesity and obesity related diseases. Studies have recognised the coexistence of two major sources of obesity: the genetically related obesity and the diet related obesity. Among the

promoters of obesity associated to diet, fat are of grate concern, first of all because they furnish more calories pr gram than the other nutrients. Dietary lipid level influences the rate of lipogenesis. Early studies showed that *de novo* synthesis of fatty acids is decreased by high dietary lipid levels (Bortz et al., 1963; Romsos and Leveille, 1974). Two key enzymes in the lipogenic pathway, i.e. fatty acid synthetase and acetyl CoA carboxylase, are reduced in animals receiving a high-fat diet. In addition, the pentose phosphate pathway and malic enzyme, both providing reducing equivalents for *de novo* lipogenesis, are also influenced by dietary lipid level. Malic enzyme, the pentose phosphate pathway and the rate-limiting enzyme in this pathway, i.e. glucose-6-phosphate dehydrogenase, are decreased in rats fed a diet containing high levels of dietary fat while they are increased in diets high in carbohydrates (Clarke, 2000). But the inhibition of lipogenesis is not uniformly influenced by different fatty acids. For example, unsaturated fatty acids are better at inhibiting *de novo* lipogenesis than saturated fatty acids are (Flick et al., 1977; Pan et al., 1990). The inhibitory effects of unsaturated fatty acids are further influenced by fatty acid chain length, degree of unsaturation and double-bond location (Grossman, 2002). There is considerable evidence that both hormone-stimulated lipolysis and the antilipolytic effects of insulin are influenced by the quantity and/or type of dietary fat. However, the effect of dietary fat on lipolysis varies somewhat according to the species and specific adipose tissue depot being studied (Grossman, 2002).

Lipoprotein lipase (LPL) has been called the ‘‘gate keeper’’ enzyme because it controls the rate of uptake of lipid by adipose cells (Greenwood, 1985). This enzyme is elevated in association with genetic and diet-induced obesity in animals and humans. The ability of *n*-3 polyunsaturated fatty acids to lower serum triglycerides is thought by some to be due to an action on lipoprotein lipase. The reduced obesity associated with diets high in *n*-3 polyunsaturated fatty acids may also be due to the influence of these fatty acids on reducing hepatic fatty acid syntase activity (Benhizia et al., 1994) and stimulating fatty acid oxidation. Although the evidence is not totally consistent, it appears that the rate of lipolysis may be influenced to some extent by both the level and the type of dietary fat. A reduction in lipolytic rate as is commonly observed in response to high-fat (particularly saturated fat) feeding could lead to an increased retention of stored triglycerides and thereby contribute to the development of obesity. However, this response may vary considerably according to species, age, adipose tissue depot site, and adipose tissue fatty acid composition. The relative contribution of lipolytic alterations to diet-induced obesity and the specific regulatory components of the lipolytic signal transduction cascade influenced by alterations in the level or type of dietary fat remain to be elucidated (Greenwood, 1985).

In a recent review, St-Onge and Jones (2002) concluded that medium chain fatty acids (MCFA) are readily oxidized in the liver as indicated by animal and human studies that have shown that the fast rate of oxidation of MCFA leads to greater energy expenditure. Furthermore, most animal studies have also demonstrated that the greater energy expenditure with MCFA relative to long-chain fatty acids (LCFA) results in a less body weight gain and decreased size of fat depots after several months of consumption (St-Onge and Jones, 2002; Marten et al., 2006).

I-2-3 Biosynthesis pathway of essential fatty acids in animals and plants

Lipids have diverse and essential roles in plants. Being the hydrophobic barrier of the membranes, they are essential for integrity of cells and organelles. In addition, they are a major form of chemical energy storage in seeds and are now recognized as a key component of some signal transduction pathways. Most lipids, but not all, contain fatty acids esterified to glycerol, and consideration of this area of metabolism involves, firstly, the synthesis of the fatty acid and, secondly, the synthesis of lipid after esterification of the fatty acid to form phosphatidic acids. In mammals and humans, they are important in the membrane composition of the different organs and also in the production of essential compounds of the biological system. In mammals, metabolites of polyunsaturated fatty acids (PUFA) are also used as signaling molecules. Arachidonic acid is abundantly stored within the cell membrane and is required as a substrate for eicosanoid synthesis (Sprecher, 1981). Eicosapentanoic acid (20:5, see the nomenclature in table 2) is the precursor of prostaglandins, while docosahexaenoic acid (22:6) is essential for nervous system maintenance and development (Marszalek and Lodish, 2005). Because of these multiple roles of unsaturated fatty acids, the ratio of unsaturated fatty acids to saturated fatty acids is strictly regulated to maintain cellular homeostasis in each organism or tissue (. Fatty acid biosynthesis in plants is very similar to that in bacteria and is carried out in the plastid. In plants, each reaction is catalyzed by a separate gene product in contrast to fatty acid synthesis in animals, which depends on a multifunctional protein. The synthesis of the major PUFA in plants and animals is represented in Figure 9.

Figure 8: Synthesis of essential PUFA in plants and animals (Hashimoto et al., 2006)

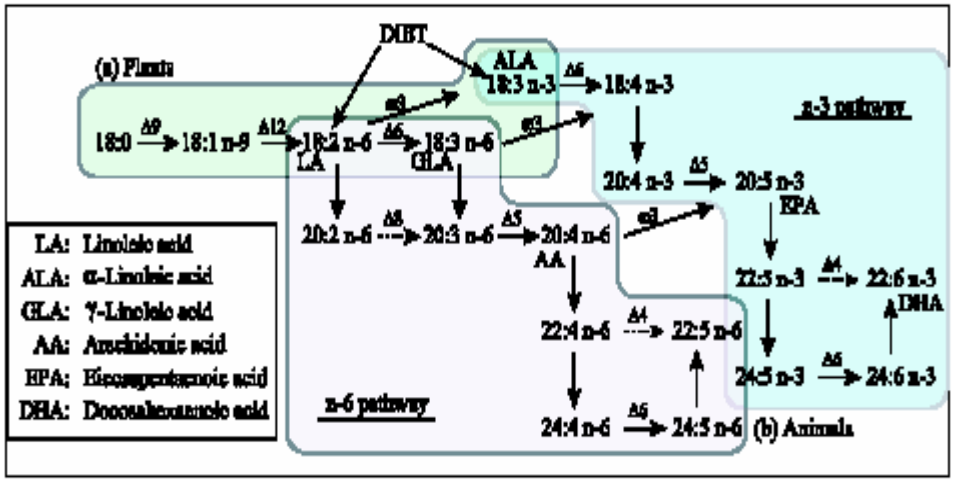


Figure 8:To designate an individual fatty acid within a family of structurally related acids, the n- nomenclature is used (see table 2). Here, the position of the first double bond from the methyl end is described e.g. 18:3 n-6 (GLA) indicates that the double bond closest to the methyl end is 6 carbons from the methyl group and in the Δ12 position. The n-6 pathway and the n-6 family are composed of n-6 fatty acids. (a) Plants generally synthesize the LA and GLA using Δ9, Δ12, and Δ15 (ω3) desaturases. (b) Animals generally synthesize AA, EPA, and DHA with Δ5 and Δ6 desaturases starting from LA and ALA obtained from the diet. Dashed lines indicate alternative pathways with Δ4 or Δ8 desaturases.

The Acyl-ACP, Δ12, Δ15, Δ5, and Δ6 desaturases listed in Table 5 are required for the synthesis of major PUFAs, such as linoleic acids, arachidonic acids, docosahexaenoic acids. The range of biosynthesis of fatty acids that each organism is capable of is different in each organism; hence, to investigate the fatty acid variation in each organism, a reference pathway of fatty acid biosynthesis, which is integrated over multiple species, has been constructed (Figure 8). In plants, acyl-ACP, Δ9, Δ12, and Δ15 desaturases introduce double bonds into the 9th, the 12th, and the 15th carbon, respectively (Napier et al., 1999). This is followed by the synthesis of linoleic acids and α-linoleic acids (Figure 8 (a)). Similarly to previous studies, the results obtained by Hashimoto et al., (2006) support both acyl-ACP and Δ12/Δ15 desaturases being present in higher plants (table 4). In general, animals take linoleic acids and α-linoleic acids synthesized by plants in the diet, and then Δ5/Δ6 desaturases introduce double bonds into them to synthesize other multivalent unsaturated fatty acids.

Table 4: Number of desaturase related to proteins in Eukaryotes (Hashimoto, 2006)

	organism	total	ACP	Δ12/15	Δ5/6	Δ9	Δ4s	com	other
Mammal	*Homo sapiens*	8	0	0	3	2	2	0	1
	Pan troglodytes	7	0	0	2	2	2	0	1
	Macaca mulatta (d)	13	0	0	4	5	2	0	2
	Mus musculus	16	0	0	3	7	5	0	1
	Rattus norvegicus	10	0	0	3	3	3	0	1
	Canis familiaris	9	0	0	3	2	2	0	2
	Bos taurus (p)	11	0	0	6	2	2	0	1
	Sus scrofa (p)	1	0	0	0	1	0	0	0
	Monodelphis domestica (d)	8	0	0	1	2	3	0	2
Bird	*Gallus gallus*	9	0	0	4	2	2	0	1
Amphibian	*Xenopus laevis* (p)	6	0	0	2	1	3	0	0
	Xenopus tropicalis (p)	3	0	0	0	1	2	0	0
Fish	*Danio rerio* (d)	7	0	0	1	3	2	0	1
	Fugu rubripes (d)	5	0	0	0	2	2	0	1
	Tetraodon nigroviridis (d)	5	0	0	0	2	1	0	2
Ascidian	*Ciona intestinalis* (d)	4	0	0	0	1	2	1	0
Insect	*Drosophila melanogaster*	7	0	0	0	6	1	0	0
	Drosophila pseudoobscura (d)	7	0	0	0	6	1	0	0
	Anopheles gambiae (d)	13	0	0	0	11	2	0	0
	Apis mellifera (d)	25	0	0	0	25	0	0	0
	Bombyx mori (d)	9	0	0	0	8	1	0	0
Nematode	*Caenorhabditis elegans*	9	0	2	0	3	2	2	0
	Caenorhabditis briggsae (d)	10	0	2	0	3	2	3	0
Plant	*Arabidopsis thaliana*	26	8	6	0	9	1	2	0
	Medicago truncatula (d)	5	0	2	0	1	1	1	0
	Oryza sativa japonica (d)	22	9	11	0	0	1	1	0
	Cyanidioschyzon merolae	4	0	1	0	2	1	0	0

	organism	total	ACP	Δ12/15	Δ5/6	Δ9	Δ4s	com	other
Fungi	*Saccharomyces cerevisiae*	1	0	0	0	1	0	0	0
	Saccharomyces paradoxus (d)	1	0	0	0	1	0	0	0
	Saccharomyces mikatae (d)	1	0	0	0	1	0	0	0
	Saccharomyces bayanus (d)	1	0	0	0	1	0	0	0
	Ashbya gossypii	5	0	1	0	1	1	1	1
	Kluyveromyces lactis (d)	6	0	2	0	1	1	1	1
	Kluyveromyces waltii (d)	6	0	2	0	1	1	1	1
	Debaryomyces hansenii (d)	6	0	2	0	1	1	1	1
	Candida albicans	7	0	2	0	1	1	1	2
	Candida glabrata (d)	1	0	0	0	1	0	0	0
	Yarrowia lipolytica (d)	4	0	1	0	1	1	1	0
	Schizosaccharomyces pombe	2	0	0	0	1	1	0	0
	Neurospora crassa (d)	6	0	2	0	1	1	1	1
	Magnaporthe grisea (d)	6	0	2	0	1	1	1	1
	Gibberella zeae (d)	8	0	2	0	1	1	2	2
	Aspergillus nidulans	8	0	2	0	2	1	1	2
	Aspergillus fumigatus	8	0	2	0	2	1	2	1
	Aspergillus oryzae	11	0	2	0	4	1	3	1
	Cryptococcus neoformans JEC21	4	0	1	0	1	1	1	0
	Cryptococcus neoformans B-3501A (d)	4	0	1	0	1	1	1	0
	Ustilago maydis (d)	4	0	1	0	1	1	1	0
	Encephalitozoon cuniculi	0	0	0	0	0	0	0	0
Protist	*Dictyostelium discoideum*	7	0	0	0	3	1	3	0
	Plasmodium falciparum	1	0	0	0	1	0	0	0
	Plasmodium yoelii (d)	1	0	0	0	1	0	0	0
	Plasmodium berghei (d)	2	0	0	0	2	0	0	0
	Plasmodium chabaudi (d)	1	0	0	0	1	0	0	0
	Cryptosporidium parvum	0	0	0	0	0	0	0	0
	Cryptosporidium hominis	0	0	0	0	0	0	0	0
	Theileria annulata	0	0	0	0	0	0	0	0
	Theileria parva	0	0	0	0	0	0	0	0
	Trypanosoma brucei	4	0	1	0	1	1	1	0
	Trypanosoma cruzi	9	0	2	0	1	2	2	2
	Leishmania major	12	0	3	0	2	4	3	0
	Entamoeba histolytica	0	0	0	0	0	0	0	0
	Giardia lamblia (d)	0	0	0	0	0	0	0	0

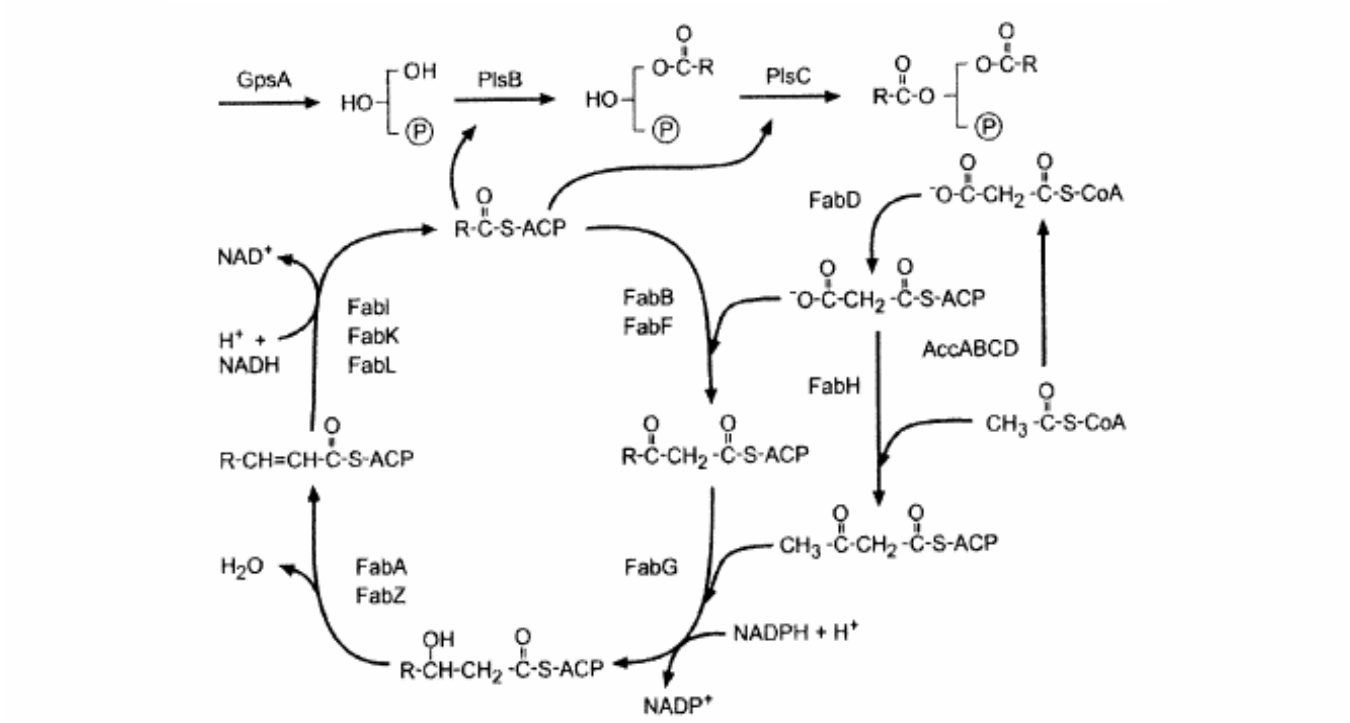
ACP : acyl-ACP desaturases in G6 group
Δ12/15 : Δ12 desaturases or Δ15 desaturases in G2 or G3 groups
Δ5/6 : Δ5 desaturases or Δ6 desaturases in G1 group
Δ9 : Stearyol-CoA desaturases in G8 or G9 groups
Δ4s : Δ4 sphingolipid desaturases in G4 group
com : variety of desaturases in G5 group
other : hypothetical proteins in G10 group
(d) : draft genome
(p) : partial genome
Non-zero values are colored for convenience

I-2-4 Biosynthesis of fatty acids in Bacteria

The biosynthesis of fatty acids for the formation of membranes is an energy-intensive and vital facet of cell physiology. Bacteria and plants accomplish this task using a highly conserved collection of enzymes called the type II fatty acid synthase system. This process differs from the mammalian type I system, which is a hard-wired process where all of the reactions are carried out at active sites located on the same polypeptide chain (Rock and Jackowski, 2002). Fatty acids produced by different bacterial groups are numerous and their diverse generation pathway are still to be totally understood. The type II fatty acid synthase system is composed of a collection of individual enzymes that are each encoded by a unique gene. The individual steps in the pathway are shown in Figure 9 and each is identified with the respective gene that is known to encode the

activity (Table 5). The central player in the pathway is acyl carrier protein (ACP), a low molecular weight protein that carries all the intermediates as thioesters attached to the terminus of its 4'-phosphopantetheine prosthetic group. The prosthetic group sulfhydryl is the only thiol group in ACP and is attached to the protein via a phosphodiester linkage to a serine (Ser-36 in *E. coli*) located in an extended loop between the first and second helices (Xu et al., 2001). The solution structure of ACP determined by NMR spectroscopy shows that it exists as a monomeric 4-helix bundle (Xu et al., 2001; Kim and Prestegard, 1989). The interpretation of the biochemical data with respect to the ACP structure suggests that the growing acyl chain is housed within a hydrophobic pocket adjacent to Ser36. However, it will be important to determine additional structures that contain the acyl chain to substantiate these predictions (Rock and Jackowski, 2002).

Figure 9: Individual steps in the pathway of bacteria fatty acid synthesis
(Rock and Jackowski, 2002).



Type II fatty acid synthesis. Acetyl-CoA carboxylase is a heterotetramer and the subunits are encoded by the *accA, accB, accC* and *accD* genes. The malonate group is transferred from CoA to ACP by malonyl-CoA:ACP transacylase (FadD). Cycles of fatty acid elongation are initiated by the condensation of acetyl-CoA with malonyl-ACP catalyzed by β-ketoacyl-ACP synthase III (FabH). The second step in the elongation cycle is carried out by β-ketoacyl-ACP reductase (FabG). The β-hydroxyacyl-ACP intermediate is dehydrated by either FabA or FabZ to form *trans*-2-enoyl-ACP. FabA is also responsible for the isomerization of the double bond at the 10-carbon stage to form unsaturated fatty acids (Fig. 2). The final step in elongation is catalyzed by enoyl-ACP reductase. There are three forms of this enzyme (FabI, FabK, and FabL). FabI and FabL are NAD(P)H-dependent reductases. FabK is a NADH-dependent flavoprotein that also oxidizes the cofactor in the absence of substrate. Subsequent rounds of elongation are initiated by the elongation condensing enzymes (FabB and FabF) whose substrate specificities govern the structure and distribution of fatty acid products. The acyl-ACP end products of fatty acid synthesis are used for the formation of membrane phospholipids. Glycerol phosphate is formed by shunting dihydroxyacetone phosphate from glycolysis by GpsA. The acyl group of long-chain acyl-ACP is transferred to the 1-position of glycerol-phosphate by the glycerol-3-phosphate acyltransferase (PlsB). The second step in the formation of phosphatidic acid is the acylation of the 2-position by the acylglycerol-3-phosphate acyltransferase (PlsC).

Table 5: Genes and enzymes of type II fatty acid synthases.

Gene	Enzyme activity
accABCD	Acetyl-CoA carboxylase
accA	Carboxytransferase subunit
accB	Biotin carboxy carrier protein
accC	Biotin carboxylase
accD	Carboxytransferase subunit
acpP	Acyl carrier protein (ACP)
fabA	β-Hydroxydecanoyl-ACP dehysdratase
fabB	β-Ketoacyl-ACP synthase I
fabD	Malonyl-CoA:ACP transacylase
fabF	β-Ketoacyl-ACP synthase II
fabG	β-Ketoacyl-ACP reductase
fabH	β-Ketoacyl-ACP synthase III
fabI	Enoyl-ACP reductase I
fabK	Enoyl-ACP reductase II
fabL	Enoyl-ACP reductase III
fabZ	β-Hydroxyacyl-ACP dehydratase II
fadR	Transcriptional activator/repressor
farR	Transcriptional activator/repressor
fabR	Transcriptional activator/repressor

The first committed step in fatty acid biosynthesis is catalyzed by acetyl-CoA carboxylase (Acc) that forms malonyl-CoA from acetyl-CoA (Figure 9). Acc is a heterotetramer that is encoded by four individual genes that encode the respective subunits (Table 5). The only known metabolic destination of malonyl-CoA is fatty acids and the key position of Acc in the formation of this intermediate makes it an obvious candidate as a regulator of the pathway. AccABCD expression correlates with the growth rate of the cell (Li and Cronan, 1993), and increased levels of AccABCD expression lead to elevated rates of fatty acid production (Davis, Solbiati and Cronan, 2000), demonstrating that transcriptional regulation is an important mechanism for controlling fatty acid synthesis. Malonyl-CoA:ACP transacylase (FadD) transfers the malonyl group from CoA to ACP (Fig. 9). This step is essential not only to deliver malonyl-ACP for initiation, but also to supply each round of fatty acid elongation (Harder et al., 1974). There is only a single FabD isoform known and it is readily identified in all completed bacterial genomes. The enzyme utilizes a malonyl-enzyme intermediate attached to a serine residue, and the 3-dimensional structure of FabD explains the unusual reactivity of the active site serine (Serre et al., 1995). This efficient and active enzyme has no apparent regulatory function in the pathway.

β-Ketoacyl-ACP synthase III (FabH) catalyzes the condensation of acetyl-CoA with malonyl-ACP to initiate cycles of fatty acid elongation (Fig. 9). This condensing enzyme differs from the elongation condensing enzymes discussed below in that it utilizes CoA thioesters as

primers rather than acyl-ACPs (Jackowski and Rock, 1987). Its position at the beginning of the pathway suggests a regulatory role and its overexpression results in an overall shortening of the fatty acid chain-lengths (Jackowski and Rock, 1987). Accordingly, FabH is a target for the feedback inhibition by acyl-ACP and the degree of inhibition increases with increasing chain length (Heath, and Rock,1996a). FabH substrate specificity is also important in determining fatty acid structure. In bacteria that synthesize straight-chain saturated and unsaturated fatty acids, FabH uses only acetyl and propionyl-CoA (Heath and Rock,1996a). In contrast, in bacteria that produce branched-chain fatty acids, the FabH component most efficiently uses branched-chain acyl-CoA derived from amino acid catabolism (Choi et al., 2000; Butterworth and Bloch, 1970; Han et al., 1998; Smirnova and Reynolds, 2001). In most bacteria, there is only one FabH enzyme in the genome; however, in *B. subtilis* there are two fabH genes. The enzymes encoded by these genes have virtually identical properties, and it is not clear if the presence of two genes has a bphysiological relevance (Choi et al., 2000). Although FabH is thought to be an essential pathway enzyme, fabH mutants are not available and genetic verification of this hypothesis is lacking (Rock and Jackowski, 2002).

The next step in the elongation cycle is the NADPH dependent reduction of the β-ketoacyl-ACP by FabG (Fig. 9). FabG is a member of the short-chain reductase/dehydrogenase superfamily (Jörnvall et al., 1995) and has a Ser-Lys-Tyr catalytic triad. There is only a single known isozyme that catalyzes the reduction of β-ketoacyl-ACP, and FabG is an essential gene in *E. coli* (Zhang et al., 1998). The 3-dimensional structure of FabG from the plant *Brassica napas* was solved as the FabG-$NADP^+$ binary complex, and more recently, the cofactor-free FabG from *E. coli* was determined (Price et al., 2001). A comparison of these structures clearly shows that a significant conformational change occurs upon cofactor binding, and biochemical studies show that a further cooperative conformation change ensues in the FabG-NADPHACP ternary complex (Price et al., 2001). There are no known compounds that inhibit FabG activity, although this enzyme appears to be an attractive target for broadspectrum antibacterial drug development in light of its ubiquitous expression, highly conserved primary structure and essential function (Rock and Jackowski, 2002).

The next step in the elongation cycle is the dehydration of the β-hydroxyacyl-ACP to the trans-2-enoyl-ACP. There are two known isoforms of this enzyme, FabA and FabZ. Both of these enzymes efficiently catalyze the dehydration of β-hydroxyacyl-ACPs, albeit with slightly different substrate specificities (Heath and Rock, 1996b). The equilibrium for this reaction lies on the side of the β-hydroxy intermediate, therefore, cycles of elongation are pulled to completion by FabI (Heath and Rock, 1995). The key difference between these enzymes is that FabZ only catalyzes the

dehydration reactions, whereas FabA not only dehydrates, but also is capable of isomerizing the double bond in the 10-carbon intermediate as the first step in unsaturated fatty acid synthesis. Gram-positive bacteria that contain only saturated, branched-chain fatty acids have only the FabZ isoform in their genome and produce unsaturated fatty acids only under cold shock conditions by introducing a double bond into preexisting acyl chains (Angilar et al., 1998).

The final step in the elongation cycle is the NAD(P)H-dependent reduction of enoyl-ACP (Fig. 9). This step has been most extensively studied in *E. coli* where there is a single, essential NADH-dependent reductase called FabI (Bergler et al., 1994). This isoform of FabI is readily identifiable in most bacteria and plants and until recently was thought to be the only enoyl-ACP reductase in type II systems. Many members of the *Streptococcus* family do not have a gene that is related to FabI, but rather use a completely unrelated flavoprotein as an enoyl-ACP reductase II (FabK) (Heath and Rock, 2000). FabK is an NADH-dependent, FMN containing protein that performs the same function in cycles of fatty acid elongation as FabI. The reason why these organisms utilize this unique enzyme may relate to its alternate activity as a NADH oxidase in the absence of substrate, which would promote glycolysis by regenerating NAD^+. Enoyl-ACP reductase III (FabL) was discovered in *B. subtilis as* an ancillary participant in fatty acid biosynthesis (Heath et al., 2000a). This reductase is related to FabI in that it has the same spacing between the key catalytic residues as observed in FabI (Tyr-X6-Lys). Finally, the fabI gene in *Pseudomonas aeruginosa* has been inactivated by transposon insertion mutagenesis, and the cells do not have a growth phenotype (Hoang and Schweizer, 1999). Thus, there is another enoyl-ACP reductase in this organism and it remains to be determined if this is a novel protein or one of the three known isoforms (Rock and Jackowski, 2002).

I-2-5 Lipids biosynthesis as a target for antibacterial agents

Fatty acid biosynthesis is coordinately regulated with phospholipids synthesis, macromolecular synthesis and growth as part of the normal response of a bacterium to a changing environment (Heath et al., 2001). Fatty acids are the principal end products of the biosynthesis pathway and form the hydrophobic portion of the membrane phospholipids. The flexible nature of the type II system allows for diversion of intermediates to other end products, such as quorum sensors, lipids and vitamins. FabI is also inhibited *in vitro* by long-chain acyl-CoA and this mechanism may be relevant to regulating the *de novo* fatty acids synthesis when cells are grown in the presence of exogenous fatty acids, although this hypothesis has not been verified with *in vitro*

yet (Heath et al., 2001). In E. coli and other Gram-negative bacteria, membrane fluidity is controlled by modulation of the ratio of saturated to unsaturated fatty acyl chains in the memebrane phospholipids (Cronan et al., 1973; Cronan, 1975). The unsaturated fatty acids are produced via isomerization of the *trans*-2 double bon to *cis*-3 at the 10 carbon stage of the synthesis (Heath and Rock, 1996b). The FabA dehydratase catalyses this isomerization, and the *cis*-3 product is condensed with malonyl-ACP by FabB, skipping the FabI-catalysed reduction and thus maintaining the double bond (Heath et al., 2001). Both FabA and FabB are essential to this process and an inactivating mutation of either locus renders the cells auxotrophic for unsaturated fatty acids (Cronan et al., 1969). The FabF condensing enzyme is responsible for modulation of the chain length in response to temperature: at low temperature, *E. coli* makes more *cis*-vaccemic acid, and cell deficient in FabF do not undergo this adaptation (Gelmann and Cronan, 1972). Gram-positive bacteria, for example, do not make unsaturated fatty acids by *de novo* synthesis and thus do not contain homolog of FabA or FabB (Heath et al., 2001). Most Gram-positive bacteria contain *iso* or *anteiso* methyl branched-chain fatty acids instead of unsaturated straight-chain fatty acids, which, when present, are synthesized from existing saturated acyl chains via an inducible desaturase (Aguilar et al., 1998).

FabI is one of the most studied fatty acid biosynthesis in bacteria as target for drugs and antibacterial compounds. It has recently been shown that the primary antibacterial target of triclosan, and related compounds, is FabI in *E. coli* (McMurry et al. 1998; Heath et al. 1998). Identification and characterization of FabI from *S. aureus* (Heath et al., 2000b) have confirmed that the antibacterial target of triclosan in this organism is also FabI (Heath et al. 2000b). However, FabK, the enoyl-ACP reductase described above and found in other organisms, is refractory to inhibition by triclosan (Heath, and Rock, 2000b). Therefore, the mode of action of triclosan against organisms in which FabI is not essential is likely to be via an alternative mechanism, possibly disruption of bacterial membranes. To date, experimental inhibitors have demonstrated that FabI and the initiating and chain-elongation condensing enzymes (FabH, and FabB and FabF, respectively) are viable antibacterial targets. It is likely that the other pathway components are also attractive antibacterial targets. Cerulenin inhibits the condensation reaction in the pathway that is responsible for the chain-elongation phase of fatty acid biosynthesis. This compound possesses similar potency in this reaction in both mammals and bacteria (Omura, 1981). On the other hand, thiolactomycin is a selective inhibitor of the bacterial condensation enzymes (Magnuson et al., 1993) (FabB, FabF and FabH), although it is a much less potent inhibitor of FabH.

Also fatty acids, in particular polinunsaturated fatty acids, are inhibitors of FabI as recently proved by Zheng et al. (2005) who found that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis, which has served as a promising target for antibacterial drugs. Additional unsaturated fatty acids including palmitoleic acid, oleic acid, linolenic acid and arachidonic acid also exhibited the inhibition of FabI. However, neither the saturated form (stearic acid) nor the methyl ester of linoleic acid inhibited FabI.

Access to a broad spectrum of bacterial genomes has played a key role in enabling a thorough assessment of this pathway as a source of novel antibacterial targets. There is already substantial proof that several of these enzymes are well-validated, essential and broad-spectrum antibacterial targets. The development of genomics-based antisense and inducible-promoter technologies will provide exquisite tools for the further evaluation of the Fab targets and could provide an insight into which enzyme(s) offer the greatest promise as antibacterial targets (Payne et al., 2001).

I-3 Exogenous fatty acids interaction with bacteria

I-3-1 How fatty acids cross the cell wall and cell membrane

The bacterial cell wall is a unique structure that surrounds the cell membrane. Although not present in each bacterial species, the cell wall is very important as a cellular component. Structuraly, the wall is necessary for:

- ✓ Maintaining the cell's characteristic shape: the rigid wall compensates for the flexibility of the phospholipid membrane and keeps the cell from assuming a spherical shape
- ✓ Countering the effects of osmotic pressure: the strength of the wall is responsible for keeping the cell from bursting when the intracellular osmolarity is much greater than the extracellular teichoic osmolarity
- ✓ Providing attachment sites for bacteriophages: teichoic acids attached to the outer surface of the wall are like landing pads for viruses that infect bacteria
- ✓ Providing a rigid platform for surface appendages: flagella, fimbriae, and pili all emanate from the wall and extend beyond it

The cell walls of all bacteria are not identical. In fact, cell wall composition is one of the most important factors in bacterial species analysis and differentiation. There are two major types of walls: Gram-positive and Gram-negative.

The cell wall of Gram-positive bacteria consists of many polymer layers of peptidoglycan connected by amino acid bridges. A schematic diagram provides the best explanation of the structure. The peptidoglycan polymer is composed of an alternating sequence of N-acetylglucosamine (NAG) and N-acetyl-muraminic (NAMA) acid. It's a lot easier to just remember NAG and NAMA. Each peptidoglycan layer is connected, or crosslinked, to the other by a bridge made of amino acids and amino acid derivatives. However, the particular amino acids vary among different species. The crosslinked peptidoglycan molecules form a network which covers the cell like a grid. Also, 90% of the Gram-positive cell wall is comprised of peptidoglycan.

The cell wall of Gram-negative bacteria is much thinner, being comprised of only 20% peptidoglycan. Gram-negative bacteria also have two unique regions which surround the outer plasma membrane: the periplasmic space and the lipopolysaccharide layer. The periplasmic space separates the outer plasma membrane from the peptidoglycan layer. It contains proteins which destroy potentially dangerous foreign matter present in this space. The lipopolysaccharide layer is located adjacent to the exterior peptidoglycan layer. It is a phospholipid bilayer construction similar to that in the cell membrane and is attached to the peptidoglycan by lipoproteins. The lipid portion of the LPS contains a toxic substance, called Lipid A, which is responsible for most of the pathogenic affects associated with harmful Gram-negative bacteria. Polysaccharides which extend out from the bilayer also contibute to the toxicity of the LPS. The LPS, lipoproteins, and the associated polysaccharides together form what is known as the outer membrane. The cell wall have no function of regulation and selectivity regarding the penetration and expulsion of material in and out of the cell respectively. This role is carried out by the cell membranes.

Bacterial cytoplasmic membranes are both functionally and structurally diverse. Cytoplasmic membranes define cells from the external environment, contain the cytoplasm and other cellular constituents. They regulate the movement of substances entering or exiting cells and catalyze exchange reactions. Membranes also play a role in energy transduction and in the maintenance of ion and solute gradients to maintain a constant intracellular environment. They also provide a milieu where biological reactions can occur and act to regulate cellular growth and metabolism. Cytoplasmic membranes stabilize protein structure, which is important with regard to the function of membrane embedded enzymes. In addition, membrane embedded molecules and receptors add to

the role of the membrane by allowing it to provide for intercellular communication and detection of cellular signals (Denich et al., 2003).

The penetration of fatty acids into the membrane depends on many factors related to the physical and chemical properties of both the membrane and the fatty acids. In an experiment done by Langner et al (1995) using two different model phospholipids bilayers (egg-phosphatidylcholine (egg-PC) and egg-phosphatidylethanolamine (egg-PE)) to evaluate the partitioning of free fatty acids (FFA), they obtained the following results:

i- at high surface concentration of the FFA, decreasing pH at the bilayer surface caused the protonation of FFA (oleic and lauric acids), and raised the pK of FFA at the bilayer surface from 5 to about 7.

ii- The partition of FFA in egg-PE vesicles was an order of magnitude lower than that of egg-PC vesicles.

Still according to the Authors, the incorporation level of the FFA in the bilayers was determined more by the molecular packing than by the nature of lipid head groups. Hamilton and Cistola (1986) showed that in a system composed of albumin and egg-phosphatidylcholine vesicles, the partitioning of oleic acid towards the vesicle was favoured by lower pH. Fatty acids can also affect the permeability of membranes. Langner and Hui (2000) observed that free fatty acids reduce the lipid bilayer permeability to ditionite and iodide ions at the main phase transition of 1,2-dimyristoyl-*sn*-glycero-3-phosphocoline bilayer. In particular, the permeability of the bilayer was reduced drastically in the presence of oleic acid, while stearic and the methyl ester of oleic acid had little effect. Banchio and Gramajo (1997) also observed that the uptake of palmitate in *Streptomyces coelicolor* A3(2) was lower when the bacteria was in the presence of oleic acid respect than when in the presence of hexadecanoic, dodecanoic, decanoic, octanoic hexanoic and butyric acids.

Regarding the mechanism of permeability of fatty acids into the membranes, the literature is very controversial, especially when concerning long chain fatty acids. Regarding FA with number of carbon lower than 12, its relatively straightforward to show that they diffuse through the membrane in an uncharged form (Hamilton, 1999). Because of the complexities of FA transport in a cell, it is useful to consider three fundamental steps of the membrane-related transport in a simplified model, using phospholipids bilayers without proteins (Hamilton, 1999). In these types of bilayers, the membrane transport of FA is distinguished from the uptake, which may include metabolic steps. To enter a cell, the FA must first adsorb to the leaflet to which it is presented, and then it must pass through the apolar interior of the bilayer to the opposite leaflet. To perform most of its functions in

the cell, the FA must desorb from the inner leaflet. A protein or the proteins could in principle be involved in any one or a combination of these individual steps, particularly if the rate of these steps is slow (Hamilton, 1999). In a recent review on this topic, Kamp and Hamilton (2006) gave the following conclusions, entirely reported in order not to introduce elements of misinterpretation.

"We have argued that FA cross protein-free phospholipids bilayers very rapidly ($t_{1/2}$<1 s). They also cross the plasma membrane of cells rapidly both in intact cells and in isolated membrane vesicles (Guo et al., 2006). In the context of the lipid phase of biological membranes, where FA bind with high affinity and rapid kinetics, and where the energy barrier for translocation of the uncharged carboxyl is low (Pownall and Hamilton, 2003), putative long chain fatty acids (LCFA) transport proteins do not fulfil classic requirements for transport proteins.

If fatty acids cross cell membranes mainly by diffusion, how can uptake into cells be controlled? Firstly, in the living organism with circulating albumin, LCFA can diffuse out of the cell if they are not utilized. No specialized mechanism is required, and the efflux follows the concentration gradient. Secondly, metabolism is a dominant regulator of FA uptake into cells. According to our hypothesis based on data from adipocytes (Kamp et al., 2003; Civelek et al., 1996) and HepG2 cells (Guo et al., 2006), the return of intracellular pH is controlled by factors affecting intracellular metabolism of the LCFA. In organs such as the placenta and the brain, where PUFA requirements, particularly ω3 FA, are higher than for other tissues, the greater enzymatic activity of activation and esterification of these FA will result in higher retention in the constituent cells. This is a mechanism for ''selective uptake'' of FA into cells and would explain observations for instance in the perfused placenta where a preferential transfer of docohexaenoic acid relative to other fatty acids was found (Haggarty et al., 1997; Larque et al., 2003).We would argue that even if proteins were important in translocating FA across the membrane, it is unlikely that they would be highly selective since well-characterized FA binding proteins such as FABP and albumin are very indiscriminate in their binding of LCFA.

Finally, membrane proteins that bind FA can help regulate FA uptake by mechanisms other than transporting FA through the lipid bilayer. One could imagine that biological membranes are engineered in such a way that the diffusion could be modulated by membrane proteins that can bind many LCFA with high affinity. LCFA cross the membrane by free diffusion (flip-flop), yet the flux would be modulated by proteins. In fact, any protein in the membrane could modulate the partitioning and/or ionization properties of FA in the membrane, particularly if the protein has a high affinity for FA. Recent studies have revealed alternative mechanisms by which membrane

proteins can regulate FA uptake. The clearest example is the family of fatty acid transport proteins (FATP). Originally postulated to move the FA across the impermeable lipid bilayer (Schaffer and Lodish, 1994), this family has now been shown to catalyze the activation of LCFA to acyl-CoA on the inner leaflet of the plasma membrane (Watkins et al., 1999; Pei et al., 2004). This removes the unesterified FA and re-establishes the concentration gradient that favours influx of FA. The second example is caveolin-1, another putative FA transporter that is localized on the cystosolic leaflet of the plasma membrane. On the basis of fluorescence experiments that monitored the adsorption and the translocation of FA in membranes on cells with different levels of expression of caveolin-1, it has been postulated that this protein provides additional binding sites for the FA anion (Meshulam et al., 2006). This property leads to an altered distribution of the FA across the lipid bilayer, favouring the inner leaflet, and may act to restrict the lateral diffusion of the FA, which could buffer the lipid bilayer against high concentrations of FA and/or promote localized metabolism of the FA."

I-3-2 Fatty acids requirements for bacterial growth

There are few evidences of fatty acids as essential nutrient requirement for most of the bacteria related to food. Only some lactic acid bacteria (LAB) and bacteria present in animal rumen are reported to need FAs for their growth. The bacterial membranes of lactobacilli are typically composed of straight-chain saturated, unsaturated and cyclopropane fatty acids (CFAs; Johnsson et al., 1995). The fatty acid composition of the bacterial membrane depends upon a number of factors, including growth temperature, pH, growth phase, cultivation medium composition and NaCl concentration (Corcoran et al., 2007). When LAB are grown in a medium supplemented with Tween 80 [polyoxyethylenesorbitanmono-oleate, which consists of up to 90% oleic acid; Partanen et al., 2001], oleic acid is incorporated into the membranes (Johnsson et al., 1995). Tween 80 is routinely included in synthetic media for cultivation of lactobacilli, where it improves aerobic growth rates (Jacques et al., 1980), glucosyltransferase secretion (Jacques et al., 1985) and glycine-betaine accumulation in LAB (Guillot et al., 2000). In addition, it has been reported that both lactobacilli and bifidobacteria are able to form dihydrosterculic acid [*cis*-9,10-methyleneoctadecanoic acid; cyc 19 : 0(9c)] via methylation (Johnsson et al., 1995; Veerkamp, 1971) and these fatty acids, also called CFAs, have been associated with enhanced bacterial acid resistance (Budin-Verneuil et al., 2005; Chang & Cronan, 1999). The presence of FA in the media do not have the same effect on the growth of different LAB species. For example, Partanen et al. (2001) reported that among the six *L. delbrueckii* strains studied all except one strain required Tween 80 or Tween 20 as a fatty acid

supplement for the growth. Tween 40 and Tween 60, which contain solely medium and long chain saturated fatty acids, inhibited the growth of all *L. delbrueckii* strains when present as a sole fat supplement in MRS broth. Free oleic acid but not free lauric acid could substitute Tween 80 or Tween 20 supplement suggesting that unsaturated fatty acids are essential growth factors for most *L. delbrueckii* strains. Among the natural food oils tested, Partanen et al. (2001), the oils containing the lowest amounts of saturated long chain fatty acids promoted the growth of *L. delbrueckii* most effectively. Especially cellular C18:1 and C19 cyclopropane fatty acid contents of *L. delbrueckii* were strongly affected by exogenous fatty acid composition and by strain suggesting genetic diversity and polymorphism among the genes encoding and/or regulating cyclopropane synthase.

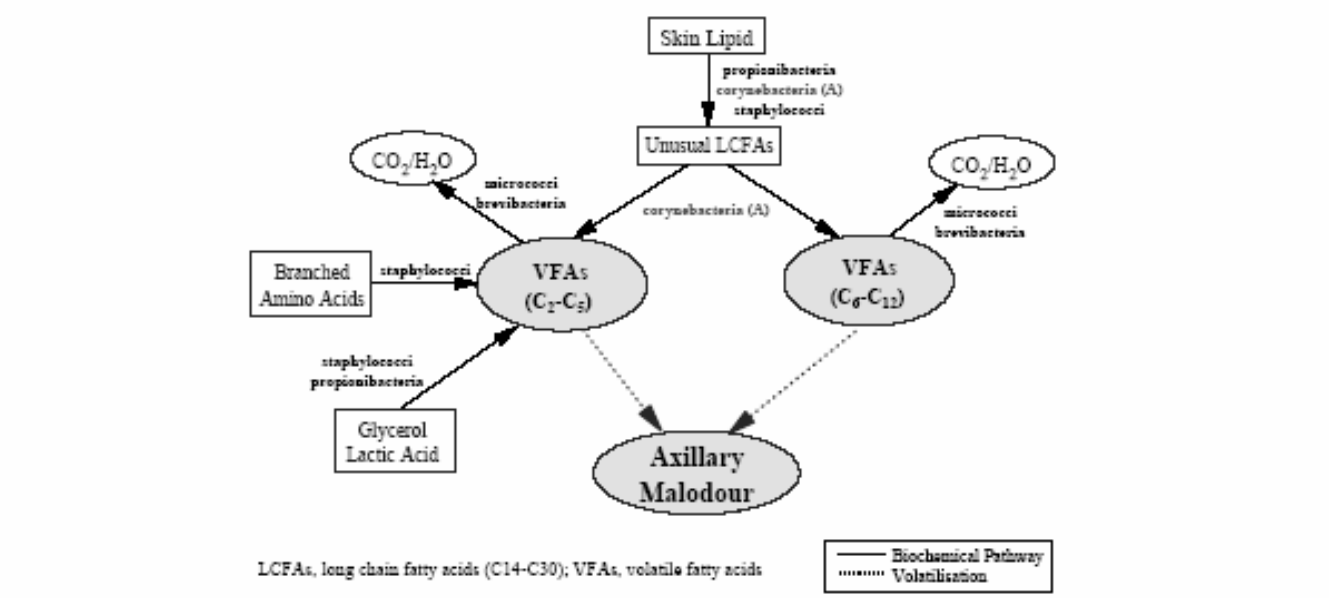
Among the lactic acid bacteria, particular attention is reported on the probiotics. They are live microbials, beneficial to health, as they can influence the microbial balance of host and modulate the host immunity. For their survival in food and during gastric transit, an important trait for probiotics is their acid tolerance (Holzapfel & Schilinger, 2002), and also their physicochemical surface properties (Kankaanpää et al., 2004). In this regards, Kankaanpää et al. (2004) observed that when lactobacilli were cultured in MRS broth supplemented with various free PUFA, the incorporation of a given PUFA into bacterial fatty acids was clearly observed. Moreover, PUFA supplementation also resulted in PUFA-dependent changes in the proportions of other fatty acids; major interconversions were seen in octadecanoic acids (18:1), their methylenated derivatives (19:cyc), and CLA. Also intermittent changes in eicosapentaenoic acid proportions were noted. These results were paralleled by minor changes in the hydrophilic or hydrophobic characteristics of lactobacilli, suggesting that PUFA interfere with microbial adhesion to intestinal surfaces through other mechanisms. In conclusion, they demonstrated that free PUFA in the growth medium induce changes in bacterial fatty acids in relation to the regulation of the degree of fatty acid unsaturation, cyclization and proportions of CLA and PUFA containing 20 to 22 carbons.

Regarding other bacteria species, some of the microrganism colonizing the rumen are stimulated by the presence of fatty acids. Maczulak et al. (1981) observed that the growth of *Selenomonas ruminantium*, *B. ruminicola* and one strain of *B. fibrisolvens* was stimulated by the presence of oleic acid.

On the other hand bacteria can use fatty acids present in the medium not only to promote growth and/or modify their membrane composition, but also to produce other compounds. This attitude of metabolizing the fatty acid is present in many microrganism. Cutaneous bacteria role in axillary malodour has been correlated to the fatty acid metabolism (James et al., 2004; Figure 9).

Also, conversion of individual and mixed volatile fatty acids to hydrogen by *Rhodopseudomonas capsulate* was reported by Shi and Yu (2006) as well as the biohydrogenation of C18 unsaturated fatty acids to stearic acid by a strain of *butyrivibrio hungatei* from the bovine rumen (Van de Vossenberg and Joblin, 2003). Another transformation of fatty acids is observed during oxidation of fat in food, specially in meat products (Chizzolini et al., 1998; Kanner, 1994, Sado et al., 2005).

Figure 10: Formation and utilization of volatil fatty acids (VFAs) by axillary bacteria. (James at al., 2004)



I-3-3 Antimicrobial activity of fatty acids

Fatty acids are naturally occurring compounds with little or no human toxicity, that can possess antimicrobial properties. Fatty acids and their esters, mainly mono and diglycerides, have been used as preservatives in some foods (Kabara, 1993;Isaacs et al., 1995; Hinton and Ingram, 2000). According to the available literature, The antimicrobial activity of these compounds depends on their physical and chemical properties and the type of microrganism. In general fatty acids function as anionic surface agents, and the anionic surfactants are less potent at physiological pH values (Scherff and Peck, 1959). The microbicidal effects of medium-chain free fatty acids and their corresponding monoglycerides have been extensively studied in recent years, mainly in

pharmacological sector. They have been found to have a broad spectrum of microbicidal activity against enveloped viruses and various bacteria *in vitro* (Kabara, 1978; Shibasaki and Kato, 1978; Welsh et al., 1979; Thormar et al., 1987; Isaac et al., 1994), including pathogens such as herpes simplex virus (HSV) (Thormar et al., 1987; Kristmundsdóttir et al., 1999), *Neisseria gonorrhoeae* (Bergsson et al., 1999) and *Chlamydia trachomatis* (Bergsson et al., 1998). These lipids are commonly found in natural products, for example in milk, and are therefore likely to be non-toxic to the mucosa, at least at low concentrations. In nature, e.g. in milk and on the mucosa, these compounds are considered to be potent inhibitory factors against infections by many human pathogens or parasites (Isaac et al., 1994; Isaacs et al., 1995). The largest study still remain that of Kabara et al. (1972), where 30 straight chain fatty acids and derivatives were tested against 8 gram-negative and 12 gram-positive organism. From their experiment, Kabara et al. (1972) concluded that C12 (lauric acid) was the most inhibitory saturated fatty acid against gram-positive organisms. Monoenoic acid (C18:l) was more inhibitory than saturated fatty acids, but was less active than dienoic derivatives (C18:2). Other unsaturated compounds were less active than C18:2. Alcohols and glyceryl esters were active only against gram-positive organisms. In general, esterification of the carboxyl group led to a compound that was less active; monoglycerides were the sole exception. Amine derivatives, contrary to results with fatty acids, esters, and amides, showed activity against both gram-positive and gram-negative organisms. Polyunsaturated fatty acids in general have demonstrated to have a higher bactericidal activity than monounsaturated and saturated (except lauric acid) fatty acids. Giamarellos-bourboulis et al. (1998) found that gamma-linolenic and arachidonic acids interacted bactericidally on *P. aeruginosa* isolates, inducing the development of strains resistant to β-lactans and to aminoglycerosides. In an experiment on real food sample, Hinton and Ingram (2000) found that oleic acid was able to reduce the number of bacteria on the skin of processed broilers, and that the fatty acid was bactericidal to several spoilage and pathogenic bacteria associated with poultry. In particular, *Campylobacter* sp., *Enterococcus faecalis* and *Listeria monocytogenes* isolates possessed the least resistance, *Escherichia coli* and *Pseudomonas aeruginosa* higher resistance while *Enterobacter cloacae*, *Staphylococcus lentus* and *Salmonella typhimurium* had the greatest resistance. Lee et al. (2002) tested the antimicrobial effect of linolenic acid with or without monoglyceride (glycerol laurate or glycerol myristate) against six food-borne microorganisms in broth medium. Minimum inhibitory concentrations of linolenic acid on *Bacillus cereus* and *Staphylococcus aureus* were 20 and 50 ppm, respectively. The growth of *B. cereus* treated with linolenic acid at 10 ppm with 10 ppm monoglycerides was more inhibitory than that of

linolenic acid alone, and the viable cell population was reduced by 2-4 log cycles compared to that of the control. Notwithstanding these results, further research is still needed on this topic, as literature lacks data on antimicrobial activity of these compounds in food environments or food-like environment. Moreover, the mechanism of action of the different fatty acids (Zheng et al., 2005) and the reasons of their activity mostly on Gram-positive than on Gram-negative are still not well understood.

I-4 Meat as source of PUFA for human diet

I-4-1 PUFA enrichment of meat

In rich societies, consumers increasingly attach importance to all those aspects that improve their quality of life. Traditionally, meat has been considered an important ingredient in the Western food culture and it has been associated with ‘‘the proper meal’’. Meat is an important provider of protein and iron and has therefore been considered essential for good health. However, during the last half of the twentieth century diseases connected to lifestyles have increased in the Western world. The relationship between constituents in the diet and health has been established, especially between saturated fat in animal products, illness and weight (Kubberød et al., 2002). The general nutritional and health implications of fats and oils is an area of research and development where the information is frequently contradictory. However, there are a number of proven facts as regards fat intake as discussed previously. Clearly, limitations in fat intake refer not only to the amount of fat but also to the fatty acid composition and the cholesterol levels in foods, of which meat and meat products constitute a major part. All fats do not have the same metabolism, and therefore the extent to which the composition of meat and meat derivatives should be modified is closely linked to cholesterol levels and fat intake (and the fatty acid pro-file). Meat fat content can vary widely depending on various factors such as species, feeding, cut, degree of separation of the fat in the various handling phases (processing of the carcass, cutting, preparation of commercial cuts, removal by the consumer), cooking conditions, etc (Nez-Colmenero et al., 2001). The lipid content in edible lean meat today is less than 5% (Chizzolini et al., 1999), so it can no longer be considered an energy-rich food. However, this is not the case of some of the leading commercial meat products, where the percentages can be as high as 40–50% and structural disintegration is so great that the consumer cannot reduce the high fat content. Fatty acid composition has a considerable effect on the diet/health relationship, since each fatty acid affects the plasmatic lipids differently. Meat lipids

usually contain less than 50% saturated fatty acids (SFAs of which only 25–35% have atherogenic properties), and up to 70% (beef 50–52%, pork 55–57%, lamb 50–52%,
chicken 70%, rabbit 62%) unsaturated fatty acids (monounsaturates, MUFAs, and polyunsaturates, PUFAs; Romans et al., 1994). The presence of MUFAs and PUFAs in the diet reduces the level of plasma low-density lipoproteins cholesterol, although PUFAs also depress the high density lipoproteins-cholesterol (Mattson & Grundy, 1985). Hence, it does not seem reasonable to describe meat generally as a highly saturated food, especially in comparison with some other products (e.g. some dairy products) as it can be observed in Table 6 (Rule et al., 2002).

Increasing the polyunsaturated fraction of meat seams to be the only way of enhancing the fat quality. In this perspective, the most suitable way a part from the genetic selection is without doubt the modification of the diet fat composition. In the last decades, introduction of fish fat or vegetable oil in place of animal fat traditionally used in animal diet has gained a lot of consideration. Corino et al. (2002), analysing the effects of dietary fats on meat quality and sensory characteristics of heavy pig loins, found that animals fed diets containing tallow, corn oil or rapeseed oil from 25 kg up to 160 kg live weight found no differences between dietary treatments for loin weight, pH, or color of longissimus lumborum (LL) muscle at 45 min and 24 h after slaughtering. No significant differences in moisture, total protein or total lipid content of LL muscle were also found. On the contrary, the linolenic acid content of the total lipid of LL muscle was higher in pigs fed rapeseed oil than those fed tallow and corn oil. Linseed is also an excellent source of omega-3 fatty acids, particularly a-linolenic acid, which are currently of interest in both human and animal nutrition (Wood and Enser, 1997; Doreau and Chilliard, 1997). Mitchaothai et al. (2006) in another experiment studied the effects of dietary beef tallow versus sunflower oil on meat quality and fatty acid composition of various tissues. They observed that fat type had no significant effect on carcass traits (carcass weight, back-fat thickness, fat-lean ratio) and meat quality (colour, pH_1, pH_U, drip losses, cooking losses, shear force, sacromere length, loin moisture, loin marbling). On the other hand, diet with sunflower oil instead of beef tallow significantly increased the incorporation of polyunsaturated fatty acids in adipose tissues, loin and liver at the expense of the sum of saturated and monounsaturated fatty acids. In erythrocytes, the diet containing sunflower oil raised the contents of saturated and polyunsaturated fatty acids and lowered that of monounsaturated fatty acids. In particular, the sunflower oil diet produced an increase in the content of linoleic acid (C18:2n-6) in the various tissues. Enser et al. (2000) also observed in a similar experiment the increase of PUFA and even of eicosapentaenoic (EPA) and docosahexaenoic acid in adipose tissue.

Table 6 Weight percentage of fatty acids[a] and concentrations of cholesterol and total fatty acids in longissmus dorsi muscle of bison, beef cattle, and elk and in chicken breast (Rule et al.,2002)

	Bison		Beef cattle				
Fatty acid[b]	Range	Feedlot	Range	Feedlot	Elk	Chicken breast	SEM (n = 10)
14:0	1.58^{e}	1.47^{e}	2.03^{e}	2.66^{d}	3.84^{c}	0.48^{f}	0.20
14:1*cis*-9	0.00^{f}	0.18^{e}	0.60^{c}	0.39^{d}	0.00^{f}	0.00^{f}	0.05
¡15:0	0.27^{c}	0.01^{f}	0.19^{d}	0.04^{f}	0.03^{f}	0.09^{e}	0.02
ª15:0	0.00^{d}	0.00^{d}	0.12^{d}	0.03^{d}	0.31^{c}	0.00^{d}	0.06
15:0	3.61^{c}	2.25^{d}	1.54^{d}	0.42^{e}	4.05^{c}	2.48^{d}	0.36
¡16:0	0.02^{de}	0.14^{c}	0.16^{c}	0.10^{cd}	0.18^{c}	0.00^{e}	0.03
16:0	17.2^{e}	18.0^{e}	22.2^{cd}	25.8^{c}	23.8^{cd}	21.8^{d}	0.97
16:1*cis*-9	2.58^{e}	3.08^{de}	2.67^{e}	3.75^{de}	10.9^{c}	5.30^{d}	0.71
¡17:0	0.70^{c}	0.30^{d}	0.56^{c}	0.26^{d}	0.23^{d}	0.21^{d}	0.07
17:0	1.31^{d}	2.19^{c}	1.32^{d}	1.20^{d}	0.50^{e}	0.04^{f}	0.09
17:1*cis*-9	1.22^{d}	2.26^{c}	1.26^{d}	1.05^{d}	0.42^{f}	0.74^{e}	0.09
18:0	16.8^{c}	12.6^{d}	13.4^{d}	13.5^{d}	8.75^{e}	8.83^{e}	0.53
18:1*trans*	0.16^{d}	0.01^{e}	0.14^{d}	0.01^{e}	0.29^{c}	0.36^{c}	0.05
18:1*cis*-9	30.7^{e}	43.3^{c}	37.5^{d}	40.4^{cd}	12.9^{f}	28.1^{e}	1.52
18:1*cis*-11	0.47^{e}	0.00^{e}	0.37^{e}	0.00^{e}	5.72^{c}	2.55^{d}	0.20
18:2*cis*-9,12	7.81^{de}	6.75^{e}	4.10^{f}	3.11^{f}	10.1^{d}	17.0^{c}	0.83
18:2*cis*-9, *trans*-11	0.34^{d}	0.28^{e}	0.41^{c}	0.26^{e}	0.10^{f}	0.07^{f}	0.02
18:2*trans*-10, *cis*-12	0.02^{d}	0.01^{f}	0.12^{c}	0.01^{de}	0.03^{de}	0.00^{f}	0.01
18:2*cis*-10,12	0.07^{cde}	0.04^{e}	0.10^{c}	0.04^{e}	0.06^{de}	0.09^{cd}	0.01
18:3*cis*-6,9,12	0.00^{e}	0.00^{e}	0.18^{c}	0.00^{e}	0.02^{d}	0.00^{e}	0.003
18:3*cis*-9,12,15	2.81^{c}	0.41^{f}	1.48^{e}	0.22^{f}	2.13^{d}	0.45^{f}	0.17
18:4*cis*-6,9,12,15	0.18^{c}	0.08^{d}	0.10^{d}	0.05^{e}	0.10^{d}	0.16^{c}	0.01
20:1*cis*-11	0.00^{d}	0.00^{d}	0.14^{c}	0.00^{d}	0.00^{d}	0.00^{d}	0.01
20:2*cis*-11,14	0.14^{d}	0.06^{e}	0.07^{e}	0.05^{e}	0.07^{e}	0.36^{c}	0.01
20:3*cis*-8,11,14	0.07^{e}	0.05^{e}	0.09^{de}	0.02^{f}	0.11^{d}	0.16^{c}	0.01
20:4*cis*-5,8,11,14	2.46^{d}	1.86^{de}	1.47^{ef}	0.79^{f}	3.82^{c}	4.69^{c}	0.29
20:5*cis*-5,8,11,14,17	1.07^{d}	0.40^{ef}	0.62^{e}	0.13^{f}	1.44^{c}	0.18^{f}	0.12
22:0	0.24^{d}	0.10^{e}	0.19^{d}	0.08^{e}	0.20^{d}	0.56^{c}	0.03
22:1*cis*-13	0.34^{e}	0.22^{e}	0.39^{de}	0.24^{e}	0.57^{d}	1.19^{c}	0.06
22:2*cis*-13,16	0.10^{d}	0.00^{e}	0.20^{c}	0.02^{e}	0.10^{d}	0.00^{e}	0.01
22:4*cis*-7,10,13,16	0.12^{d}	0.11^{d}	0.07^{d}	0.10^{d}	0.09^{d}	1.05^{c}	0.03
22:5*cis*-7,10,13,16,19	1.25^{c}	0.53^{de}	0.71^{d}	0.26^{e}	1.31^{c}	0.31^{e}	0.10
22:6*cis*-4,7,10,13,16,19	0.23^{c}	0.18^{d}	0.09^{e}	0.04^{f}	0.11^{e}	0.26^{c}	0.01
24:0	0.04^{d}	0.01^{e}	0.01^{e}	0.01^{e}	0.01^{e}	0.15^{c}	0.01
Unknown-<C16	1.94^{d}	0.53^{e}	0.74^{de}	0.33^{e}	3.75^{c}	0.05^{e}	0.40
Unknown-C16-18	2.26^{d}	1.16^{e}	3.17^{c}	0.41^{f}	2.39^{d}	1.00^{e}	0.17
Unknown->C18	1.97	1.44	1.52	4.25	1.70	1.24	1.24
SFA	41.7^{c}	37.0^{d}	41.7^{c}	44.0^{c}	41.9^{c}	34.7^{d}	1.00
PUFA	16.5^{d}	10.7^{e}	9.53^{ef}	5.04^{f}	19.4^{d}	24.6^{c}	1.43
P/S	0.40^{de}	0.29^{ef}	0.23^{fg}	0.12^{g}	0.49^{d}	0.71^{c}	0.05
$n-3$	5.35^{c}	1.51^{e}	2.90^{d}	0.64^{e}	5.00^{c}	1.19^{e}	0.38
$n-6$	10.3^{e}	8.66^{ef}	5.66^{fg}	3.92^{g}	14.0^{d}	21.9^{c}	1.08
$n-6/n-3$	1.94^{e}	5.73^{d}	1.95^{e}	6.38^{d}	2.84^{e}	18.5^{c}	0.35
Total fatty acids, mg/100 g	11.1^{e}	20.7^{d}	10.7^{e}	28.8^{c}	8.05^{e}	7.94^{e}	1.90
Cholesterol, mg/100 g	43.8^{e}	54.1^{d}	52.3^{d}	52.7^{d}	50.2^{d}	59.3^{c}	1.23

[a]Weight percentage values are relative proportions of all peaks observed by GLC.

[b]Fatty acids are represented as number of carbon atoms:number of carbon–carbon double bonds. Unknowns were peaks presumed to be fatty acids of the carbon lengths indicated based on retention times relative to known fatty acids. P/S ratio is the ratio of polyunsaturated fatty acids (PUFA) to saturated fatty acids (SFA). $n-3$ fatty acids included 18:3*cis*-9,12,15, 20:5*cis*-5,8,11,14,17, 22:5*cis*-7,10,13,16,19, and 22:6*cis*-4,7,10,13,16,19, and $n-6$ fatty acids included 18:2*cis*-9,12, 20:3*cis*-8,11,14, and 20:4*cis*-5,8,11,14.

[c,d,e,f,g]Means in a row with different superscripts are different ($P < 0.01$).

I-4-2 Microbes related to meat product

Meat can be infected with or carry a wide range of microorganism that can be considered as spoilage or potentially pathogenic. The contamination of meat is highly correlated with the hygienic conditions of the animal rearing, slaughtering and conditioning. The spoilage microflora associated with meat stored in presence of oxygen, is composed prevalently of *Pseudomonas*, *Acinetobacter* and *Psychrobacter* because of their high growth rate under this condition. *Brochothrix thermosphacta*, *Enterobacteriaceae,* psychrophilic and lactic acid bacteria because of their low growth rate in these conditions colonize the product later on. Other spoilage bacteria are *Bacillus* spp., *Staphylococcus*, *Carnobacterium*, *Corynobacter* and yeast. Aberle et al. (2001) explain that most of the microbiological contamination experienced might be overcome with vacuum-packaging, but also point out that the low levels of oxygen that are trapped in or on the meat are used by the microorganisms and result in metmyogloblin formation. These activities consequently result in carbon dioxide production and pH reduction whilst facultative anaerobic bacteria may thrive in these conditions and produce lactic acid. The consequent bacterial growth is inhibited by the low pH and oxygen absence, significantly extending the shelf life of the vacuum-packed meat. However, in the case of heavily contaminated meat it might undergo pigment decomposition, discolouration and development of off-odours resulting from the growth of certain anaerobic bacteria (Gill, 1996). Potential spoilage bacteria and pathogens generally associated with red meat include *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Campylobacter* spp and *Salmonella* spp. (Eisel et al., 1997; Forsythe, 2000; Nel et al., 2004; Humphrey and Jørgensen, 2006). As this thesis is focused only on *Staphylococcus aureus* , only this pathogen will be further developed.

I-4-3 *Staphylococcus aureus*

Staphylococcal species are divided into coagulase-positive (*S. aureus*, *S. intermedius*,..) and coagulase-negative species (*S. carnosus* and *S. xylosus*). The occurrence of *Staphylococcus aureus* on raw meat would be expected, because it is one of the principal components of the skin of humans and animals (Adams & Moss, 1997). *Staphylococcus aureus* is a Gram-positive bacteria among the most important pathogen related to food poisoning (Scherrer et al., 2004). The primary habitats of *S. aureus* are the mucous membranes of the human nasopharynx and animal skin; it is also present in soil, water sources, dust and air (Gundogan et al., 2005). The presence of *S. aureus* in foods is often related to improper handling by personnel, who are frequently contaminated with these micro-

organisms (Hatakka et al., 2000). Some strains of *S. aureus* may be isolated from healthy carriers (Ahmed et al., 1998; Suzuki et al., 1999; Tondo et al., 2000). The isolation of the same strain from a food and from certain personnel, in the same period of time, or during the investigation of an outbreak, strongly suggests that food handler may be the source of contamination. *S. aureus* can be transmitted by different foods, including milk (Çitak et al., 2003; Scherrer et al., 2004), ice cream (Kanbakan et al., 2004), beef (Schlegelova et al., 2004) and chicken (Alvarez-Astorga et al., 2002). One of the first correlations of *S. aureus* with food poisoning was described by Denys (1894), when members of a family became ill after eating *S. aureus* contaminated meat obtained from a sick cow. Olsen et al. (2000) reported that *Staphylococcus aureus* was involved in 42 documented outbreaks of food-borne poisoning in the United States, with 1413 cases and 1 death occurring from 1993 to 1997. *S. aureus* has been estimated to cause approximately 185000 illnesses, 1750 hospitalizations, and 2 deaths per year in the United States, all from consumption of contaminated foods (Mead et al., 1999). From June until July 2000 a large outbreak of SFP involving 13 420 individuals occurred in the Kansai district in Japan (Asao *et al.*, 2003). The source of intoxication was linked to low-fat milk and drink-type yogurts manufactured at an Osaka city factory. Food-borne staphylococcal poisoning, caused by the ingestion of one or more preformed toxins in food contaminated with *S. aureus*, is one of the most prevalent causes of gastroenteritis worldwide (Jablonski et al., 1997). A clinical description of SFP can be quoted from Elek (1959) Comprehensive treatise on the organism *Staphylococcus pyogenes*: "The clinical course is characteristic: symptoms generally appear in about three hours, occasionally one to six hours, after ingesting food containing enterotoxin. The incubation period depends not only on the amount of toxin consumed, but also the susceptibility of the individual. Salivation is followed by nausea, vomiting, retching, abdominal cramps of varying severity, and diarrhoea. In very severe cases blood and mucus have been observed in the vomitus. Marked prostration, headache, and sweating accompany severe attacks, and there may be fever or shock with subnormal temperature and lowered blood pressure". Death due to Staphylococcal food poisoning (SFP) is uncommon, but intoxication of particularly susceptible populations such as children and the elderly, can lead to mortalities (Holmberg and Blake, 1984; Pisu and Cavallazzi, 1951).

Staphylococcal enterotoxins (SE) also play a role in serious invasive staphylococcal disease, since in addition to eliciting gastrointestinal symptoms, SEs can act as superantigens and induce toxic shock (Banks *et al.*, 2003; Ellis *et al.*, 2003; Jarraud *et al.*, 1999). In a post-9/11 world, the potential use of the staphylococcal enterotoxins in a terrorist attack needs to be fully understood by

first responders, since the symptoms of enterotoxin inhalation would be markedly more serious than enterotoxin ingestion (Gustafson, 2005). The dose of aerosolized staphylococcal enterotoxin B (SEB) that incapacitates 50 % of the human population exposed is 0.4 ng/kg, while the lethal dose for 50 % of the humans exposed is estimated to be 20 ng/kg (Hursh *et al.*, 1995). The lethal effects of the inhaled form of the SEB are probably due to its ability to act as a superantigen, as lymphoproliferation of T cells has been observed in rhesus monkeys following SEB inhalation (Mattix *et al.*, 1995). Of nine humans who accidentally inhaled SEB, besides suffering from gastrointestinal symptoms, five individuals exhibited signs of inspiratory rales and dyspnea (shortness of breath) and seven experienced moderately intense chest pains (Ulrich *et al.*, 1997). In addition, individuals exposed to SEB exhibit conjunctivitis with localized cutaneous swelling (Rusnak *et al*., 2004). SEs belong to a large family of staphylococcal and streptococcal exotoxins collectively referred to as the pyrogenic toxin superantigens (PTSAgs). Normal antigens stimulate 1 in 10 000 T cells, whereas PTSAgs can stimulate up to 20 % of all T cells (Gustafson, 2005). This non-specific polyclonal T-cell expansion results in a massive pathological release of cytokines, which then induce the most severe symptoms of toxic shock (Gustafson, 2005). SEs induce polyclonal T-cell expansion by binding to both the major histocompatibility complex (MHC) class II molecules on target cells and T-cell receptors displaying specific β-chain variable domains (McCormick *et al.*, 2001).

SEs exhibit a high level of heat stability (Bergdoll, 1983) and environmental factors within food matrixes contribute to this heat stability (Schwabe *et al.*, 1990). For example, SEA present within mushrooms retains biological activity after being heated to 121 °C for 28 min (Anderson *et al.*, 1996). Secondly, SEs are tolerant to proteolytic cleavage by gastrointestinal proteases such as pepsin, trypsin, chymotrypsin and rennin (Bergdoll, 1983). This digestive resistance allows SEs to enter the gastrointestinal tract where they interact with suspected SE receptors initiating the symptoms of SFP. The combination of heat stability and protease resistance make SEs highly effective foodborne toxins (Gustafson, 2005). The emetic response initiation targets for SEs are believed to be located within the abdominal viscera and are thought to involve specific SE receptors (Sugiyama and Hayama, 1965).

Regarding the stress response, it can be said unlike other pathogens, that in *S. aureus* is less understood. In general, the activation of all bacterial stress response systems leads to global alterations in both the cell transcriptome and protome (Gustafson, 2005). Frequently, exposure of a microbe to one type of stress can provide cross-protection against another kind of stress (Leyer and

Johnson, 1993; Pichereau *et al.*, 2000). Exposure of bacteria to low and high temperatures has profound effects on all aspects of microbial cell structure and function. The structural integrity of macromolecules, macromolecular assemblies, protein synthesis, membrane fluidity and membrane transport are all affected. Many heat and other stress-induced proteins are molecular chaperones or proteases (Gustafson, 2005). The cold shock response refers to changes occurring in the cell in response to a sudden decrease in temperature. Cold acclimation refers to longer-term alterations in the cell in response to the re-establishment of growth at a low temperature. Cold shock proteins (Csp) are low molecular weight proteins that are heavily expressed in response to cold shock in a wide range of bacteria (Phadtare *et al.*, 2000).

Concerning acid stress, the bacterial response to low pH is comprehensive, involving both constitutive and inducible responses, including removal of protons, alkalination of the environment, cell envelope composition changes, and production of stress proteins and associated transcriptional regulators. This topic in Gram-positive bacteria has been reviewed by Cotter and Hill (2003). *S. aureus* is killed at pH 2, but is protected from killing by pre-exposure to pH 4 via a *sigB*-dependent mechanism (Chan *et al.*, 1998). Adaptation of *S. aureus* to acid also induces *sodA* which encodes the major superoxide dismutase, and *sodA* mutants demonstrate reduced acid tolerance (Clements *et al.*, 1999).

Lithgow *et al.* (2004) also demonstrated a role for cysteine synthase in the acid tolerance mechanism of *S. aureus*.

Sodium chloride and sucrose are impermeant solutes that impose an osmotic stress on bacteria. Growth in the presence of high concentrations of NaCl also results in extensive alterations in gene and protein expression in *S. aureus*, many of which are not obviously related to osmoregulation (Vijaranakul *et al.*, 1997). Bacteria respond to osmotic stress by elevating their internal concentrations of compatible solutes, either by accumulation of the solutes from the medium, or by biosynthesis (Gustafson, 2005). A compatible solute is one that has little or no inhibitory effect on cell metabolism, structure or growth at high concentrations. The cellular level of K+ is high in *S. aureus* (> 1 M), and does not change much upon osmotic stress, in contrast to several other bacterial species. Various compatible solutes such as glycine-betaine, proline, choline, taurine, proline-betaine and carnitine act as osmoprotectants and accumulate via various transport systems in osmotically stressed *S. aureus* (Graham and Wilkinson, 1992; Vilhelmsson and Miller, 2002). The ability of osmoprotectants to protect bacterial viability is dependent on the substance used to reduce *a*w (Gustafson, 2005). For instance, when the permeant humectant glycerol was used

to lower medium *a*w, growth of *S. aureus* was not enhanced by osmoprotectants (Vilhelmusson and Miller, 2002).

Regarding the antibiotic resistance of *S. aureus*, the first evidence of *S. aureu*s resistance to penicillin appeared in 1941, only 2 years after its introduction in clinical therapy (Pesavento et al., 2007). Genes expressing antibiotic resistance in *S. aureus* can be either in chromosomic or in plasmidic DNA. In the case of plasmidic resistance, *S. aureus* cannot conjugate with another cell because of the lack of the ‘‘sexual pilus’’, that is usually present in Gram-negative bacteria. In such a condition, fagic transduction or membrane binding are necessary (Courvalin, 1994). Penicillin resistance is plasmidic, and therefore it spread out very quickly to several other strains, with the result that in the 1980s approximately 90% of *S. aureus*, isolated from patients, were resistant. Unlikely, methicillin-resistance is chromosomic, and therefore its diffusion is slower than the former, but it keeps going (Pesavento et al., 2007). The increase of antibiotic resistant *S. aureus*, is mostly related to the misuse of antibiotics in the treatment of human diseases, animal farming and agriculture. Probelms of human health regarding *S. aureus* resistance to antibiotic is related in prevalence to methicillin and vancomycin. This pathogen is also resistant to a large number of other antibiotics. In fact, Gundogan et al. (2005) analysing *S. aureus* strains isolated from meat and chicken, observed that their overall methicillin resistance rate for was 67.5%. and 87.5% *S. aureus* strains were resistant to bacitracin. A high prevalence of penicillin G resistance was detected for *S. aureus* (53.8%). Few of the strains were resistant to erythromycin (7.5%). All strains were susceptible to vancomycin, sulbactam–ampicillin, ciprofloxacin and cefaperazone–sulbactam. In humans, the prevalence of methicillin-resistant *S. aureus* (MRSA) largely depends on the region, site of infection, and whether the infection was nosocomial or community onset (Acco et al., 2003) In Europe, prevalence of MRSA is increasing: from 0.1–1.5% for Denmark, Sweden and The Netherlands, to 30.3–34.4% for Spain, France and Italy (Voss et al., 1994) Vancomycin is the antimicrobial commonly used in case of MRSA infection. In 1997, Hiramatsu et al. (1997) described the first clinical *S. aureus* isolate with intermediate resistance to vancomycin. Since then, other strains of *S. aureus* with reduced sensitivity to vancomycin were identified in Europe and in USA (CDCP, 1997). The possibility of transmission of MRSA through food was unknown until 1994 (Kluytmans et al., 1995); however, today we know that when a few cells of *S. aureus* enter an immunocompetent organism, they are destroyed by the gastric juices, but when an immunocompromised patients (food) contains cells of *S. aureus*, these can reach the circulatory system and cause infections that may evolve to septicaemia (Pesavento et al., 2007). This high

resistance of *S. aureus* to many drugs is the reason why one of the main goal today is the search of new bactericidal compounds or drugs for *Staphylococcus aureus* to which they have not yet develop resistance and that can possibly not induce to resistance.

I-5 References chapter I

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Chapter II

OBJECTIVES

Consumers interest in the quality aspects of food is increasing with the globalization and high rate of information diffusion. Any new development in food production is followed by a massive media advertisement which have a high impact on public opinion. One of the products that have focused the attention of consumers in the recent years is meat. Some aspects like the outbreak of bovine spongiform encephalopathy (BSE), *Salmonella* in chicken (arising from the use of clenbuterol) and dioxin-contaminated animal feed in Belgium have caused a lack of confidence within the consumers. Another negative fact related to meat is the implication of certain of its constituents in some of the most prevalent diseases in the rich societies like cardiovascular disease, hypertension, cancer and obesity. According to the World Health Organization (WHO), saturated fats should not provide more than 10% of the calories in the diet. As discussed previously in the introduction, meat fat content can be subjected to variation, depending on various factors such as species, feeding, the type of cut and the degree of fat separation during processing. Genetics and feeding are the two most applied strategies chosen to ameliorate the fatty acid composition of meat products. Regarding the meat feeding strategies, in the last decades, an attempt in the modification of the meat fatty acid composition trough the variation of the animal fodder fat source has been made. In fact the inclusion of vegetable fats in place of animal fats in the diet has already shown good results in chicken and pigs, increasing the meat polyunsaturated fat content and the omega 3 /omega 6 fatty acids ratio.

One of the common law of natural equilibrium is that any change in the system causes the formation of a different equilibrium and hence new adaptation facilities or difficulties for the living organisms. The introduction of the polyunsaturated fat source in the animal diet and consequently the modification of animal meat fatty acid composition creates a different meat environment. This variation of the meat system need to be studied in all the aspects related to food nutrition, stability and safety in order to assess the advantages and/or disadvantages of the new product. Together with the raw material composition, the transformation and storage technique can contribute to the nutritional, chemical and safety stability of the product.

In the first part of this thesis, rabbits (chosen for their high growth rates and also for their high production in Italy) were fed with diets containing different percentages of linseed as source of polyunsaturated fatty acids, with the following aims.

i- Assess the fatty acid variation of rabbit meat as a function of the level of enrichment of their diet with a source of polyunsaturated fatty acids

ii- Assess the influence of meat fatty acid composition and the atmosphere of storage on the microbial and chemical spoilage pattern during the refrigerated storage of the rabbits minced meat.

The complexity of real system based experiments increases the difficulties of correlating the observed results with changes in the system environment because of the large number of variability that are not taken into account. For these reasons, more focused investigations were carried out *in vitro* in order to reduce the system variability and complexity. In particular, attention was paid particularly on selected free fatty acids present during meat storage and their effect on the microflora. Another simplification was introduced with the choice of a single bacteria for the study, represented by *Staphylococcus aureus*, known as one of the most frequent pathogen of meat products.

In the second part of the thesis, *Staphylococcus aureus* was grown in some of the conditions observed during fresh and cured meat storage, obtained by combining different pH and incubation temperatures in broth medium. Selected fatty acids at different concentrations were added in the medium in order to:

i- Assess the minimal inhibitory concentration of the selected free fatty acids in the different conditions tested

ii- Assess the probability of total deactivation of *Staphylococcus aureus* as function of pH, temperature of incubation, free fatty acid concentration and inoculum level

The presence of these free fatty acids in food can be a result of an addition or a consequence of endogenous and microbial enzymatic activities. Generally, the concentration of the FFAs is lower than the observed minimal inhibitory concentration assessed. The question that arises is the possible effects of such concentrations on the microbial population. In this third part of the thesis, *Staphylococcus aureus* was used as a target microrganism and the effect of non-inhibitory concentrations of selected free fatty acids evaluated with the following main aims:

i- The study of the effect of non-inhibitory concentrations of selected free fatty acids on the growth rate of *Staphylococcus aureus*

ii- The assessment of the influence of non-inhibitory concentrations of selected free fatty acids on the *Staphylococcus aureus* single cells and population lag time

The different magnitude of action of the free fatty acids observed on the microrganism is an indication of a possible variation in their mechanism. In literature, some hypothesis are presented regarding this aspect although some of them, like the interaction with the fatty acids biosynthesis, have not been well established yet. In the last part of the thesis, an attention was deserved to the macroscopic evidences of the modality of action of some selected fatty acids at non inhibitory concentrations on *Staphylococcus aureus* with the following aims:

i- Assessment of the free fatty acids effect on the extracellular release of esterase by *Staphylococcus aureus*

ii- Assessment of the relation between the exogenous free fatty acids and the *de novo* fatty acid synthesis in *Staphylococcus aureus*

Chapter III

INFLUENCE OF DIETARY LINSEED AND STORAGE ATMOSPHERE ON THE SHELF LIFE AND SPOILAGE PATTERNS OF RABBIT MINCED MEAT PRODUCTS

III-1 Introduction

Literature regarding the human health benefits of n-3 polyunsaturated fatty acids (PUFA) has stimulated interest in increasing the n-3 PUFA level in animal products. The dietary supplementation is achieved either with fish oils (Ishida et al., 1996) or a source of eicosapentaenoic and docosahexanoic precursors or other ingredients containing α-linolenic acid (Ahn, Lutz, & Sim, 1996). The interest in improving the nutritional quality of food by means of manipulation of the fatty acid composition of animal feeds has also been used to improve the fatty acid profile of carcass fat in pigs (Morgan et al., 1992; Vanoeckel & Boucque, 1992) and of meat products (Bosi et al., 2000; Hoz et al., 2004). In monogastric animals such as rabbit the ratio of unsaturated to saturated fatty acids (FAs) in the fat can be changed with the diet also to improve the nutritional quality of the meat (Cobos et al., 1993; Hernandez et al., 2000; Dal Bosco et al., 2004). Dal Bosco et al. (2004) established the ability of rabbit to synthesise long chain n-3 PUFA from precursors via the elongase and desaturase pathways. In particular, the conjugated linoleic acids enrichment of rabbit meat resulted in an improvement of meat texture and tenderness (Corino et al., 2004). However, the PUFA of meat polar lipids were reported to be the primary substrate for the generation of oxidation reaction products and distinctive flavour characteristics of the meat (Grau et al., 2001). In order to prevent the off-flavour products, some studies on rabbit (Lopez-Bote et al., 1997; Castellini et al., 2001; Dal Bosco et al., 2001) have shown an improvement in tissue oxidative stability attained by feeding animals with supraoptimal levels of various antioxidants like vitamin E together with the PUFA source. Moreover, the use of modified atmosphere during storage of the products can contribute to enhance the keeping qualities and chemical stability. Although the modified atmospheres have shown not to influence the meat texture (Rubio et al., 2007), physico-chemical parameters and sensory parameters (Rubio et al., 2006), the impact of such atmospheres contribute

to modification of the original system, and hence introduce a new variable to be taken into consideration.

On the other hand little work has been done regarding the effect of dietary supplementation with a source of n-3 PUFA on the microbial quality and safety of the rabbit meat as well as on the meat microbial population and its evolution during refrigerated storage. In fact, most of the microbiological investigation, at least concerning rabbits, has been limited to the effect of fibre and protein source on the caecal microflora as well as the caeco colic rate of passage (Canzi et al., 2000; Gidenne, & Bellier, 2000; Gidenne, Jehl, Segura, & Michalet-Doreau, 2002).

In general the carcasses and refrigerated meat products are colonised by microorganisms prevalently selected by the environmental factors such as post slaughtering processes, composition of the packaging atmosphere or temperature (Abdullah et al.,1994; Bailey et al., 1993). In fact, the anaerobic species, which are prevalent in the rumen or coeco-coli-segment, do not maintain their competitive advantage in carcasses and meat products after slaughtering. The evolution during storage of the intestinal microflora, which contaminates the meat surface during slaughtering, has been analysed only in relation to pathogenic organisms such as *Escherichia coli* O157:H7 (Grauke et al., 2003; Diez-Gonzalez, Callaway, Kizoulis, & Russel, 1998). Aerobic microbial genera such as *Pseudomonds, Moraxella, Acinetobacter* are regarded as the usual colonisers of refrigerated meats. It is generally assumed that the growth dynamics of the psychrotrophic species of these genera are principally affected by temperature and packaging atmosphere composition and not by meat compositive features. However, although it is known that the diet supplementation with PUFA influences the composition and fatty acid profile and their changes during refrigerated storage in the resulting meat (Gandemer, 2002), the importance of such changes on the meat microbial population has not been previously assessed. Moreover, the spoilage patterns and the evolution of the various microbial groups in rabbit meat during refrigerated storage, also in relation to the package atmosphere and meat fatty acid composition, is still less studied.

The principal aim of this work was to evaluate whether the PUFA diet supplementation of linseed has repercussions on the free fatty acids release during storage, and on the different microbial groups growth dynamics in rabbit minced meat as well as on the accumulation of spoilage chemical markers.

III-2 Materials and methods

III-2-1 Animals, diets and samples preparation

The experiment was carried out using a total of 288 commercial rabbit hybrids, grown in intensive conditions. The animals, of the genetic type Martini TOP97® , were weaned at 30 days of life and grown in industrial conditions until the 55th day when they were divided into 4 different diet groups as indicated in the diet profiles in Table III-1. In particular, the normal commercial feed was used as control feeding, while different percentages of linseed (3%, 6% and 9%) were introduced as increasing source of polyunsaturated fatty acids in the three remaining groups. The fodders used were composed in order to maintain the same energetic and protein contain. The control fodder was characterized by the presence of palm oil (Megalac®) as lipid source, while the other experimental diets (3%, 6% and 9%) were characterized by the substitution of sunflower seed with linseed. Soya flour was the source of proteins and vitamin E was added in the form of DL-α-tocopheryl-acetate at concentration of active form equal to 200mg/Kg. At 81 days of age, the rabbits were slaughtered in an industrial plant, the meat separated from the bones and the minced meat mixtures obtained using the normal commercial plant recipes as indicated in table III-2.

From any minced meat mixture originating from animal feed with one of the diets, three equal parts were obtained and used to produces hamburgers that were stored in three different atmospheres. The different atmosphere used were: Ordinary atmosphere (OA), modified atmosphere (MA) composed of (70% O_2; 30% CO_2) and vacuum (VAC). All these combinations of diets and storage atmospheres resulted in the following samples: 0% OA, 3% OA, 6% OA, 9% OA, 0% MA, 3% MA, 6% MA, 9% MA, 0% VAC, 3% VAC, 6% VAC, 9% VAC, where 0% represented the control samples and 3%, 6% and 9% the increasing percentage of linseed in the fodders. The samples were stored at 4°C and the microbiological, physical and chemical analyses carried out over time.

Table III-1: Fodders composition for the different diets

	Diets fodders			
Fodders composition	**0%**	**3%**	**6%**	**9%**
linseed	0,0	3,0	6,0	9,0
Sunflower seed	0,0	6,0	3,0	0,0
Palm oil	2,8	0,0	0,0	0,0
Tender wheat bran	24,0	24,0	24,0	24,0
Dehydrated alfalfa flour	20,0	21,9	20,3	21,7
Hay	12,0	8,0	9,7	10,3
Sunflower extraction flour	12,0	11,7	11,0	9,5
Soya extraction flour	6,0	4,0	4,0	4,0
Barley flour	11,3	9,2	9,7	9,0
Tender wheat flour	6,0	6,0	6,0	6,0
Cane molasses	2,5	2,5	2,5	2,5
Mineral and vitamin integration	1,5	1,4	1,4	1,8
Calcium carbonate	1,1	1,7	1,8	1,4
Sodium chloride	0,5	0,5	0,5	0,5
Di-calcium phosphate	0,3	0,2	0,2	0,2
TOTAL	100,0	100,0	100,0	100,0
Chemical composition				
Protein	16,5	16,5	16,5	16,5
Total lipid [1]	5,0	5,8	5,5	5,2
Fibre [2]	15,2	15,0	15,2	15,1
Energy	2352	2369	2348	2340

[1] Antongiovanni *et al*. (1980)
[2] Folch *et al.* (1957)

Table III-2: Recipe of the minced meat production

Ingredients	**Kg**	**%**
Minced rabbit meat	23,7	79,0
Premix for Hamburger[1]	1,65	5,5
Colorant[2]	0,03	0,1
Food fibre	0,15	0,5
Water	5,07	16,9
Total	30	100

[1] Ingredients: salt, vegetable fibre, potato starch flour, dextrose, sodium citrate(E331) antioxidants (ascorbic acid, E300; sodium ascorbate, E301), spices, white pepper.
[2] Ingredients: dextrose, salt, natural vegetable extracts.

III-2-2 Microbiological analyses

Ten grams of each sample were mixed with 90 mL of 0.9 % (w/v) sterile saline solution and then homogenised for 2 min using a Lab Blender stomacher (Seward Medical, London, UK). Further serial decimal dilutions were made and then 0.1 mL of each dilution was spread onto the surface of media in triplicate. Total viable mesophilic and psychrotrophic bacterial counts were determined using plate count agar (PCA; Oxoid, Basingstoke, UK) after incubation for 48 h at 30°C and for 10 days at 4°C, respectively; Staphylococci counts were determined using Baird Parker medium with added Egg Yolk Tellurite Emulsion (Oxoid) after incubation at 37°C for 48 h. Lactic acid bacteria were enumerated by pour plating 1mL of each decimal dilutions on MRS agar with added cycloheximmide (0.012 g/L). After setting, a 10-ml of molten medium was added and the dry plates incubated at 45°C for 32 h in anaerobic jar containing H_2 and CO_2 gases (generated by an Oxoid BR38 kit). All plates were visually examined for typical colony types and morphology characteristics associated with each growth medium.

The data recorded were expressed as Log CFU/g of minced meat and the growth dynamic curves fitted to the Baranyi and Roberts, (1994) model, using the associated excel add-in DMFit freely available at http://www.ifr.bbsrc.ac.uk/safety/DMFit/default.html .

III-2-3 Total and free fatty acids analysis

Lipid extraction was based on what described by Rodriguez-Estrada et al.(1997). About 15-20g of sample was added to 200ml of a $CHCl_3/CH_3OH$ solution (1:1, v/v) and 20µl of a butyl hydroxyl toluene (BHT) solution (1,2 mg BHT / 100 µl of methanol), homogenized for 1min x 2 with an ultraturax at 18000g/min (Yellow line DI 18 basic, OPTO-LAB s.n.c. Modena, Italia) and placed in an oven for 20 min at 60°C, and then 100 ml of $CHCl_3$ was added to the mixture. This gave a final ratio of 2:1 (v/v) of $CHCl_3/CH_3OH$, which corresponds to 20 ml of solution /1 g as suggested by Folch et al. (1957). The mixture was once more homogenised for 1 min and filtered to eliminate the solid residue. The filtered fraction was added to 100 ml of 0.88 M KCl, and left overnight at 4°C in a refrigerator. The next day the chloroform phase was separated using a filter paper containing anhydrous sodium sulphate, dried in a rotovapor (Laborotta 4001-efficient, Heidolph Instruments GMBH & CO. KG Deutschland) at 40°C and the lipid extracted, weighed. The extracted lipid was conserved in hexane/ 2-propanol solution (4:1 v/v) and stored at -18°C until analysis done within two months from the extraction.

For total fatty acid composition, aliquots of the stored lipids were dried under nitrogen gas in order to have around 50mg of lipid in a tube. The free fatty acids fraction of the total lipids was first methylated at ambient temperature with 10 drops of Diazomethane (Fieser and Fieser, 1967). 1 ml of hexane and 100 µl of methanolic KOH 2N were then added and homogenized for 30s on a Vortex (Velp Scientifica Rx^3, Milano Italia). Another 1 ml of hexane was added and then homogenized again and left to separate. An appropriate quantity of the hexane phase was then diluted 10 times to a final volume of 1ml of the desired concentration of methyl esters in which 50 µl of Heneicosanoic acid methyl ester/hexane 1000 ppm (w/v) was added for GC analysis.

The separation of the free fatty acid fraction was performed by using SPE with NH_2 cartridges (International Sorber Technology Ltd, MID Glamorgan, UK) according to the method of Kaluzny et al. (1985). 50 µl of tridecanoic acid / hexane 1000ppm (w/v) was added in the lipid before the extraction to evaluate the percentage of FFA recovery, while 50 µl of Heneicosanoic acid methyl ester/hexane 1000ppm (w/v) was added before the methylation with Diazomethane (Fieser and Fieser, 1967). The GC analysis was done in the same conditions as for the total fatty acid.

A GC-FID (Perkin Elmer Autosystem XL) and a capillary column (Supelcowax-10 30m x 0.32 mm x 0.25 µm) were used for the fatty acids analyses. The injector and the detector were both held at 240°C. The temperature was programmed from 50°C (held for 1 min) to 220°C at a rate of 4°C/min and held at 220°C for 10 min. The carrier gas was helium with a rate of 1.2ml/min and the split 1:20. Fatty acids were quantified using internal satandards and identified by comparing their retention times with those of the standards mix Fame 37(Supelco, Sigma-aldrich, Milano Italy). All the solvents used in the extraction and methylation were from Carlo Erba Reagenti (Milano, Italy) except for Diazomethane that was synthesized in the Lab. Data were recorded as mean values of three different analyses per sample.

Total fatty acids were grouped into saturated fatty acid (SFA), momounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and the following nutritional index such as PUFA/SFA, n6 PUFA/ n3PUFA, were calculated. Atherogenicity index (IA) and thrombogenicity index (IT) were calculated according to Ulbricht and Southgate (1991).

Free fatty acids were analysed at 0, 4 and 7 days of storage and grouped into saturated FFA (SFFA), momounsaturated FFA (MUFFA), polyunsaturated FFA (PUFFA) and total FFA (tot. FFA).

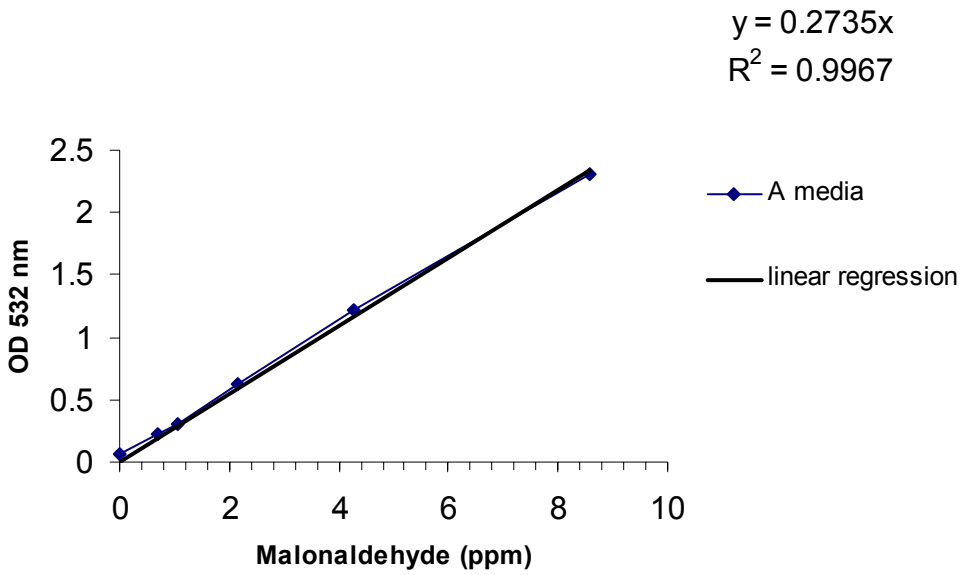
III-2-4 Volatile compounds analysis

GC-MS analyses were carried out on an Agilent 6890 gaschromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode (ionization voltage 70 eV). A Chrompack CP-Wax 52 CB capillary column (50m length, 0.32 mm inner diameter) was used. The temperature program was: 50°C for 2 min, then programmed at 1°C/min to 65°C and finally at 3.5°C/min to 220°C which was maintained for 14 min. Injector, interface and ion source temperatures were 250°C, 250°C and 230°C, respectively. Injections were performed with a split ratio of 1:20 and helium (1 mL/min) as carrier gas. Compounds were identified by using National Institute of Standards and Technology – United States Environmental Protection Agency – National Institute of Health (NIST/EPA/NIH - Version 1998) and Wiley - Version 1996 mass spectra databases and, whenever possible, by matching their retention times and mass spectra with those of standards. Head space volatile pattern were analysed at 0, 7 and 11 days of storage, and the results expressed as area.

III-2-5 Thiobarbituric acid reactive substances analyses (TBARS)

The oxidation level of the different samples was measured using the thiobarbituric acid test according to Bidlack et al., (1973). Briefly 5g of sample homogenized in 25 ml of bi-distillate water and 25 ml of trichloroacetic acid 20% for 2 min, with an ultraturax at 18000g/min. After 10 min centrifugation at 5000g, 1.5 ml was made to react with equal quantity of thiobarbituric acid 0.02 M (prepared using sulphuric acid 50 mM) for 20 min at 100°C. After cooling, the optical density (OD) of the solution was measured at 532 nm using an UV-visible spettrophotometer 1601 Shimadzu Italia S.R.L. The quantification of malondialdehyde (MAD) was done using the standard curve indicated in figure III-1 and expressed as mg of malondialdehyde per kg of sample (ppm).

Figure III-1: Standard curve of Malondialdehyde at 532 nm.



III-2-6 Denaturating gradient gel electrophoresis analysis (DGGE)

III-2-6-a Extraction of DNA from meat products

At each sampling time during storage, 10-g samples were homogenized in a stomacher bag with 10 ml of saline-peptone water for 1 minute. After each preparation had settled for 1 min, 80 ml of lytic solution (0.1 N NaOH and sodium dodecyl sulfate 1%) were added and then homogenized for 2 minutes. After setting for 30 minutes at 30°C, 1-ml sub-sample was transferred into a 1.5-ml eppendorf and centrifuged at 5500 *g* for 2 minutes. 750 µl of the supernatants were transferred into a 2-ml eppendorf and added with 150 µl of $NaClO_4$ (6M) and 900 µl of a chloroform-isoamylic alcohol (24:1) solution. After gently mixing, a centrifugation at 12000 *g* for 5 minutes was performed. The superior aqueous phases (ca 700 µl) were then collected and added with 700 µl of chloroform-isoamylic alcohol solution. The tubes were centrifuged at 12000 *g* for 10 min, the aqueous phases were collected, and the nucleic acids were precipitated with ice-cold absolute ethanol. The DNA were collected by centrifugation at 12000 *g* for 10 min and washed with 200 µl of ethanol (70%). The tubes were then centrifuged at 12000 *g* for 5 minutes, the ethanol was completely evaporated and the pellets diluted with 30 700 µl of sterile water.

III-2-6-b PCR protocol

The V1 region of the 16S rDNA was amplified with the primers P1 and P2 reported in table 1 according to Cocolin *et al*. (2001). Amplification was conducted in a standard reaction mixture (25 µl) containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1.25 U of *Taq* polymerase, and each primer (P1 and P2) at a concentration of 0.2 µM. Two microliters of template DNA was added to each mixture. Amplifications were carried out with a T 3000 Thermocycler (Biometra®, CITTA', PAESE) by using a final volume of 25 µl and the following PCR program: an initial touchdown procedure in which the annealing temperature was decreased from 60 to 52°C at a rate of 2°C every two cycles and then 20 additional annealing cycles at 50°C. A denaturation step of 95°C for 1 min was used, and extension was performed at 72°C for 2.5 min; a final extension of 72°C for 5 min ended the amplification cycle. Fifteen microliters of each PCR product was analyzed by electrophoresis in a 2% agarose gel before DGGE analysis.

III-2-6c DGGE analysis

The Dcode universal mutation detection system (Bio-Rad Laboratories, Richmond, Calif.) was used for a DGGE analysis of the PCR Electrophoresis was performed in a 0.8-mm polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]) by using a denaturant gradient from 40 to 60% (100% denaturant was 7 M urea plus 40% [wt/vol] formamide) increasing in the direction of electrophoresis. The gels were subjected to a constant voltage of 130 V for 6 h at 60°C, and after electrophoresis they were stained for 15 min in stained with ethidium bromide, rinsed in distilled water and photographed under UV illumination.

Table III-3 –PCR-DGGE primer used

Primer [a]	Sequence, 5'-3'	*position*[b]	16S rRNA gene target region	Reference
P1-f	GCG GCG TGC CTA ATA CAT GC	41-60	V1	Klijn *et al*. (1991)
P2-r	TTC CCC ACG CGT TAC TCA CC	111-130		

[a] f, forward; r, reverse; GC, a 40-bp GC clamp (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) was attached to the 5' end of the primer; [b] *Escherichia coli* numbering;

III-2-7 Statistical analyses

Statistical analyses were computed using Statistica, 6 of StatSoft, inc. USA. The significativity test for the importance of factors on the microbial counts, microbial growth and chemical parameters was performed with an ANCOVA analysis.

III-3 Result

III-3-1 Fatty acid and free fatty acids composition of the minced meat

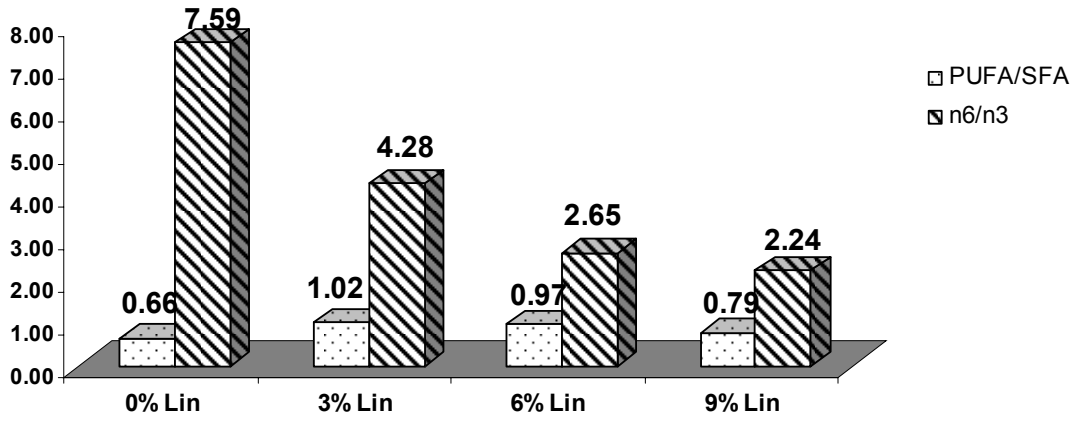
In table III-4 the fat content, expressed as total lipid percentage of the different types of samples, their centesimal composition and correlated nutritional indexes are reported. The highest fat contain was observed in the control samples with respect to those obtained from rabbit meat fed with polyunsaturated fatty acid enriched diets. Regarding the fat composition, it can be observed that in general different compositions were observed as diet changed. In fact, if we consider the fatty acid aggregates no significant differences were observed on total SFA, while total MUFA was higher when no linseed or 9% linseed was added to the diet with respect to 3% and 6% linseed supplementation. On the contrary, the total PUFA were higher in 3% and 6% diet than in the control samples and 9% diet. Among the nutritional indexes, the n6/n3 and PUFA/SFA are the most common, as they indicate the level of polyunsaturated FA and principally of the “good fat”. As reported in table III-4 and figure III-2, the n6/n3 percentage decreased with the linseed percentage increase, while PUFA/SFA was higher in presence of 3 and 6% of linseed in the diet respect to the control and 9% linseed diet.

Table III-4: Percentages of fat contain and fatty acid composition of minced meat obtained from rabbit fed with different diets

	0% Linseed	3% Linseed	6% Linseed	9% Linseed
Total lipids (% in meat)	**14±1.5**	**9.2±0.8**	**8.8±0.91**	**11.2±1.2**
C10:0 Capric acid	0.23±0.11	0.21±0.05	0.17±0.08	0.17±0.06
C12:0 Lauric acid	0.26±0.08	0.22±0.03	0.21±0.05	0.20±0.06
C14:0 Myristic acid	0.01±0	0.03±0.03	0.01±0.01	0.0
C15:0 Pentadecanoic acid	3.35±0.58	2.66±0.17	2.58±0.17	2.70±0.27
C16:0 Palmitic acid	28.54±0.03	23.56±0.17	24.52±0.06	26.31±0.12
C17:0 Heptadecanoic acid	0.72±0.41	0.60±0.57	0.32±0.21	0.50±0.64
C18:0 stearic acid	5.62±0.39	6.10±0.22	6.38±0.05	7.70±0.38
C20:0 Arachidic acid	0.11±0.32	0.09±0.15	0.03±0.40	0.07±0.02
C24:0 Nervonic acid	0.01±0.05	0.13±0	0.04±0.14	0.01±0.22
Total saturated FAs (SFA)	**38.86±1.97**	**33.60±1.40**	**34.25±1.16**	**37.65±2.77**
C15:1 *cis*-10-pentadecenoic acid	0.25±0.48	0.14±0.41	0.08±0.03	0.13±0.53
C16:1n-7 Palmitoleic acid	5.71±0.06	3.67±0.13	3.47±0.08	3.78±2.35
C17:1n-7 *cis*-10-heptadecenoic acid	0.16±0.03	0.29±0.68	0.25±0.80	0.18±0
C18:1n-9 Oleic acid	27.41±0.02	26.71±0.10	27.01±0.15	31.55±0.56
C18:1n-7 Vaccenic acid	1.35±0.09	0.58±0.10	0.65±0.42	0.06±0.35
C20:1n-9 Eicoseneic acid	0.22±0.09	0.27±0.01	0.34±0.02	0.45±0.04
Total mono unsaturated FAs (MUFA)	**35.10±0.77**	**31.66±1.43**	**31.81±1.49**	**36.14±3.82**
C18:2n-6 Linoleic acid	21.68±0.14	26.81±0.05	23.24±0.04	19.96±0.08
C18:3n-3 Linolenic acid	2.87±0.03	6.45±0.01	9.02±0.01	9.09±0.01
C20:2n-6 Eicosadienoic acid	0.15±0.12	0.18±0.16	0.18±0.04	0.23±0.01
C20 :3n-6 *cis*-8-11-14-Eicosatrienoic	0.10±0.06	0.12±0.04	0.04±0.06	0.08±0.37
C20:4n-6 Arachidonic acid	0.58±0.10	0.65±0.03	0.60±0.06	0.30±0.09
C20:5n-3.Eicosapentaenoic acid (EPA)	0.09±0.10	0.04±0	0.06±0.08	0.08±0
C22:2 Docosadienoic acid	0.09±0.01	0.01±0.02	0.19±0.03	0.13±0
C22:6n-3 Docosahexaenoic acid (DHA)	0.00	0.01±0	0.01±0.0	0.00
Total polyunsaturated FAs	**25.56±0.56**	**34.26±0.31**	**33.33±0.33**	**29.86±0.57**
Polyunsaturated/Saturated	0.66	1.02	0.97	0.79
n6/n3	7.59	4.28	2.65	2.24
Atherogenic Index	0.47	0.36	0.38	0.40
Thrombogenic Index	1.02	0.90	0.89	0.87

A more complex index introduced by Ulbricht and Southgate (1991), represented by Atherogenic and thrombogenic indexes, also indicated that the meat fat nutritional quality was enhanced by the PUFA enrichment of the animal diets.

Figure III-2: n6/ n3 and PUFA/SFA ratios as factors of linseed percentage in the animals fodders



The release of free fatty acids (FFAs) was also monitored during storage. In tables III-5a, III-5b and III-5c indicate the FFAs released at 0, 4 and 7 days of storage under ordinary (OA), modified (MA) and vacuum (VAC) atmosphere are reported, respectively. In these tables, the standard deviations have not been reported for reason of simplicity, but they have been introduced in the selected figures extracted from them (figures III-3a, III-3b, III-3c and III-4). In general, the most released free fatty acids under all the experimental conditions were palmitc, palmitoleic, oleic, linoleic and α-linolenic acids. Figures III-3a, III-3b and III-3c summarize the changes in free fatty acids aggregates as function of the linseed percentage in the animal diet, in OA, MA and VAC, respectively. The major free fatty acid groups released were the PUFFA, followed by the MUFFA and finally the SFFA regardless the linseed percentage in the diet and the atmosphere of storage. Moreover, a higher increase over time was observed for the MUFFA and PUFFA contents than for the SFFA. The quantity of FFA released in samples packed in modified atmosphere and vacuum was higher than those in ordinary atmosphere samples; moreover, samples from the 3% linseed diet showed a constant value of total FFA at the 4^{th} and 7^{th} day of storage (Figure III-4). It seems that a certain selectivity in the release of FFAs occurred during the lipolysis and that the highest percentages were represented by the PUFFA and in particular by linoleic acid followed by α-linolenic acid. The content of the former FFA in the minced meat did not depend on the percentage of linseed in the diet, while it was the case for α-linolenic acid.

Table III-5a: Free fatty acids released at 0, 4 and 7 days of storage at 4°C under ORDINARY ATMOSPHERE in sample obtained from rabbit fed with 0%, 3%, 6% and 9% linseed in their diets.

mg / Kg of sample (ppm)	0%T0	0%AO-T4	0%AO-T7	3%AO-T0	3%AO-T4	3%AO-T7	6%AO-T0	6%AO-T4	6%AO-T7
C10:0 Capric acid	0.88	1.26	2.48	0.80	1.10	1.00	0.36	0.86	1.75
C12:0 Lauric acid	1.21	1.67	2.56	0.99	1.40	1.27	0.67	1.19	1.23
C14:0 Myristic acid	0.02	0.00	0.16	0.06	0.00	0.00	0.04	0.02	0.08
C15:0 Pentadecanoic acid	5.08	7.49	12.68	4.41	6.43	5.67	3.46	6.05	8.91
C16:0 Palmitic acid	31.87	33.27	71.60	31.49	32.60	36.71	27.53	34.57	44.98
C17:0 Heptadecanoic acid	0.36	0.81	1.25	0.58	0.80	0.95	0.39	0.30	1.04
C18:0 Stearic acid	12.14	12.83	20.92	14.86	12.77	14.85	12.44	13.35	15.31
C20:0 Arachidic acid	0.68	0.02	0.35	0.41	0.10	0.22	0.74	0.03	0.16
C24:0 Nervonic acid	0.08	1.08	1.04	0.09	0.07	1.08	0.03	0.88	1.18
Total saturated FAs	**52.30**	**58.43**	**113.04**	**53.68**	**55.27**	**61.75**	**45.66**	**57.24**	**74.65**
C15:1 *cis*-10-pentadecenoic acid	0.73	0.14	0.38	0.65	0.83	0.36	0.74	0.51	0.25
C16:1n-7 Palmitoleic acid	17.05	37.82	43.75	9.52	19.72	18.02	3.10	18.84	20.10
C17:1n-7 *cis*-10-heptadecenoic acid	1.09	1.87	2.28	0.32	1.45	0.14	0.54	1.22	2.20
C18:1n-9 Oleic acid	73.57	137.50	162.78	73.18	123.62	108.87	54.16	117.59	156.29
C18:1n-7 Vaccenic acid	6.44	0.36	15.29	2.57	0.50	8.62	4.12	0.42	12.38
C20:1n-9 Eicoseneic acid	1.59	1.42	4.49	1.07	2.75	2.97	1.02	2.99	4.57
Total mono unsaturated FAs	**100.47**	**179.11**	**228.96**	**87.31**	**148.87**	**138.98**	**63.69**	**141.57**	**195.80**
C18:2n-6 Linoleic acid	84.72	184.90	213.37	94.53	189.47	180.09	59.42	169.72	215.57
C18:3n-3 Linolenic acid	15.49	29.83	31.42	30.19	60.16	56.45	30.10	89.69	111.50
C20:2n-6 Eicosadienoic acid	0.72	1.55	2.30	0.80	1.72	1.67	0.06	1.04	2.00
C20 :3n-6 *cis*-8-11-14-Eicosatrienoic	0.48	0.96	1.28	0.55	0.99	0.97	0.40	0.98	1.21
C20:4n-6 Arachidonic acid	3.15	6.48	6.62	3.62	5.63	5.88	2.06	6.51	6.59
C20:5n-3.Eicosapentaenoic acid (EPA)	0.35	0.50	0.90	0.43	0.53	1.06	0.50	1.14	1.98
C22:2 Docosadienoic acid	0.84	2.73	2.63	0.39	1.39	0.81	1.08	3.10	3.39
C22:6n-3 Docosahexaenoic acid (DHA)	0.11	0.06	0.53	0.27	0.44	0.85	0.02	0.78	1.10
Total polyunsaturated FAs	**105.86**	**227.01**	**259.04**	**130.78**	**260.33**	**247.79**	**93.64**	**272.95**	**343.33**
Total free fatty acids	**258.64**	**464.55**	**601.05**	**271.77**	**464.47**	**448.51**	**202.98**	**471.76**	**613.77**

Table III-5b: Free fatty acids released at 0, 4 and 7 days of storage at 4°C under MODIFIED ATMOSPHERE in sample obtained from rabbit fed with 0%, 3%, 6% and 9% linseed in their diets.

mg / Kg of sample (ppm)	0%T0	0%MA-T4	0% MA-T7	3% MA-T0	3% MA-T4	3% MA-T7	6%- MA-T0	6% MA-T4	6% MA-T7
C10:0 Capric acid	0.88	1.86	3.09	0.80	1.81	9.05	0.36	1.28	1.62
C12:0 Lauric acid	1.21	2.72	3.68	0.99	2.35	2.17	0.67	1.73	2.15
C14:0 Myristic acid	0.02	0.04	0.25	0.06	0.08	0.06	0.04	0.02	0.05
C15:0 Pentadecanoic acid	5.08	14.51	16.18	4.41	11.38	9.93	3.46	8.34	10.76
C16:0 Palmitic acid	31.87	67.16	77.72	31.49	58.40	17.16	27.53	43.63	55.78
C17:0 Heptadecanoic acid	0.36	1.74	1.35	0.58	1.36	1.33	0.39	1.16	1.39
C18:0 Stearic acid	12.14	16.83	20.57	14.86	19.86	16.97	12.44	15.59	18.04
C20:0 Arachidic acid	0.68	0.05	0.23	0.41	0.13	0.11	0.74	0.16	0.22
C24:0 Nervonic acid	0.08	0.54	1.28	0.09	0.90	0.11	0.03	0.41	0.08
Total saturated FAs	**52.30**	**105.45**	**124.34**	**53.68**	**96.27**	**56.88**	**45.66**	**72.34**	**90.09**
C15:1 *cis*-10-pentadecenoic acid	0.73	5.09	0.42	0.65	3.10	0.26	0.74	0.82	0.17
C16:1n-7 Palmitoleic acid	17.05	46.13	57.34	9.52	31.65	29.20	3.10	22.09	27.26
C17:1n-7 *cis*-10-heptadecenoic acid	1.09	1.68	3.27	0.32	1.24	2.24	0.54	1.98	2.35
C18:1n-9 Oleic acid	73.57	168.22	203.73	73.18	184.31	168.83	54.16	144.43	168.38
C18:1n-7 Vaccenic acid	6.44	0.17	19.59	2.57	0.10	13.14	4.12	0.33	13.68
C20:1n-9 Eicoseneic acid	1.59	4.20	5.44	1.07	3.99	4.49	1.02	3.32	4.75
Total mono unsaturated FAs	**100.47**	**225.49**	**289.79**	**87.31**	**224.39**	**218.16**	**63.69**	**172.97**	**216.59**
C18:2n-6 Linoleic acid	84.72	202.36	265.48	94.53	221.49	272.18	59.42	180.77	235.47
C18:3n-3 Linolenic acid	15.49	39.25	58.75	30.19	88.78	88.66	30.10	101.91	124.33
C20:2n-6 Eicosadienoic acid	0.72	1.75	2.34	0.80	2.44	2.49	0.06	1.69	2.14
C20 :3n-6 *cis*-8-11-14-Eicosatrienoic	0.48	0.97	0.57	0.55	1.34	1.33	0.40	1.04	1.28
C20:4n-6 Arachidonic acid	3.15	4.77	6.63	3.62	7.05	4.11	2.06	5.36	6.81
C20:5n-3.Eicosapentaenoic acid (EPA)	0.35	0.39	0.40	0.43	1.47	1.31	0.50	1.08	2.11
C22:2 Docosadienoic acid	0.84	2.14	2.75	0.39	1.04	4.34	1.08	1.36	3.12
C22:6n-3 Docosahexaenoic acid (DHA)	0.11	0.07	0.52	0.27	0.58	0.85	0.02	1.17	0.77
Total polyunsaturated FAs	**105.86**	**251.70**	**337.43**	**130.78**	**324.19**	**375.27**	**93.64**	**294.39**	**376.03**
Total free fatty acids	**258.64**	**582.64**	**751.56**	**271.77**	**644.85**	**650.31**	**202.98**	**539.71**	**682.70**

Table III-5c: Free fatty acids released at 0, 4 and 7 days of storage at 4°C under VACUUM in sample obtained from rabbit fed with 0%, 3%, 6% and 9% linseed in their diets.

mg / Kg of sample (ppm)	0%T0	0%VAC-T4	0% VAC-T7	3% VAC-T0	3% VAC-T4	3% VAC-T7	6%- VAC-T0	6% VAC-T4	6% VAC-T7
C10:0 Capric acid	0.88	0.83	1.74	0.80	2.45	4.41	0.36	1.22	3.00
C12:0 Lauric acid	1.21	1.16	1.92	0.99	2.79	2.81	0.67	1.61	3.27
C14:0 Myristic acid	0.02	0.00	0.06	0.06	0.07	0.11	0.04	0.05	0.20
C15:0 Pentadecanoic acid	5.08	6.20	10.63	4.41	15.40	13.27	3.46	8.35	11.99
C16:0 Palmitic acid	31.87	31.98	68.12	31.49	75.79	59.38	27.53	41.65	43.50
C17:0 Heptadecanoic acid	0.36	0.73	1.57	0.58	1.80	0.39	0.39	0.37	1.07
C18:0 Stearic acid	12.14	12.54	22.70	14.86	23.27	19.52	12.44	14.97	6.06
C20:0 Arachidic acid	0.68	0.12	0.35	0.41	0.29	0.04	0.74	0.17	0.60
C24:0 Nervonic acid	0.08	0.44	1.29	0.09	1.56	1.88	0.03	1.02	1.76
Total saturated FAs	**52.30**	**54.00**	**108.37**	**53.68**	**123.42**	**101.81**	**45.66**	**69.41**	**71.44**
C15:1 *cis*-10-pentadecenoic acid	0.73	0.59	0.52	0.65	1.63	0.02	0.74	1.34	0.00
C16:1n-7 Palmitoleic acid	17.05	19.77	25.22	9.52	35.99	37.87	3.10	23.13	40.23
C17:1n-7 *cis*-10-heptadecenoic acid	1.09	1.67	2.37	0.32	2.79	2.89	0.54	2.12	3.61
C18:1n-9 Oleic acid	73.57	121.90	148.59	73.18	219.55	229.45	54.16	146.79	202.56
C18:1n-7 Vaccenic acid	6.44	0.20	12.15	2.57	0.08	16.07	4.12	0.54	41.99
C20:1n-9 Eicoseneic acid	1.59	2.90	4.39	1.07	4.74	5.46	1.02	3.57	6.49
Total mono unsaturated FAs	**100.47**	**147.03**	**193.24**	**87.31**	**264.78**	**291.77**	**63.69**	**177.48**	**294.88**
C18:2n-6 Linoleic acid	84.72	162.42	215.62	94.53	323.93	345.53	59.42	195.53	325.80
C18:3n-3 Linolenic acid	15.49	86.04	116.08	30.19	102.77	110.23	30.10	105.94	175.14
C20:2n-6 Eicosadienoic acid	0.72	1.40	2.02	0.80	2.92	2.39	0.06	1.78	2.15
C20 :3n-6 *cis*-8-11-14-Eicosatrienoic	0.48	0.85	0.68	0.55	1.65	1.84	0.40	0.56	1.91
C20:4n-6 Arachidonic acid	3.15	5.53	7.68	3.62	8.67	9.63	2.06	6.38	10.30
C20:5n-3.Eicosapentaenoic acid (EPA)	0.35	1.40	2.07	0.43	1.71	2.03	0.50	1.78	3.07
C22:2 Docosadienoic acid	0.84	0.83	2.96	0.39	1.51	1.06	1.08	2.40	4.28
C22:6n-3 Docosahexaenoic acid (DHA)	0.11	0.09	0.93	0.27	0.11	1.18	0.02	0.85	2.25
Total polyunsaturated FAs	**105.86**	**258.56**	**348.04**	**130.78**	**443.27**	**473.89**	**93.64**	**315.22**	**524.90**
Total free fatty acids	**258.64**	**459.59**	**649.65**	**271.77**	**831.47**	**867.47**	**202.98**	**562.11**	**891.21**

Figure III-3a: Aggregates of free fatty acids (FFA) released during storage in Rabbit minced meat obtained from animals fed with different dietss and stored at 4°C in Ordinary atmosphere (OA).

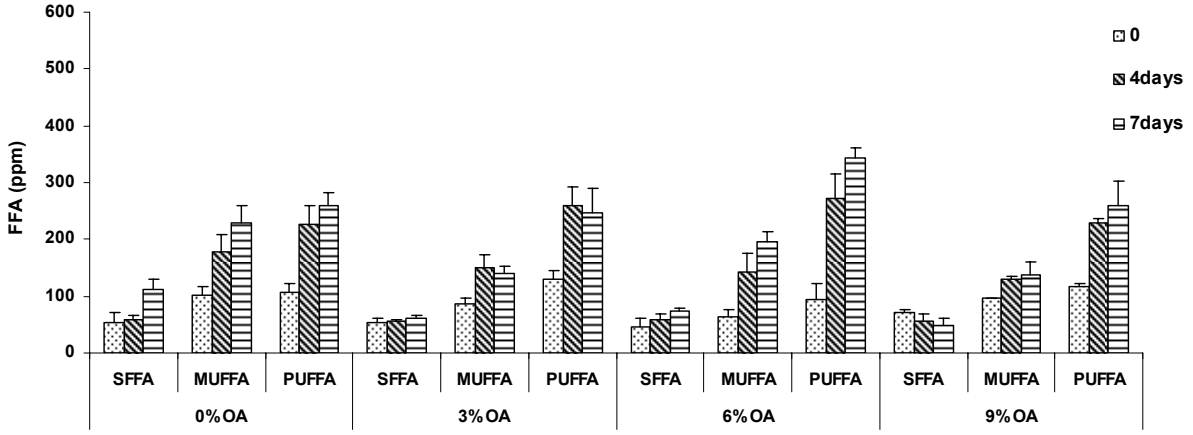


Figure III-3b: Aggregates of free fatty acids (FFA) released during storage in Rabbit minced meat obtained from animals fed with different diets and stored at 4°C in modified atmosphere (MA).

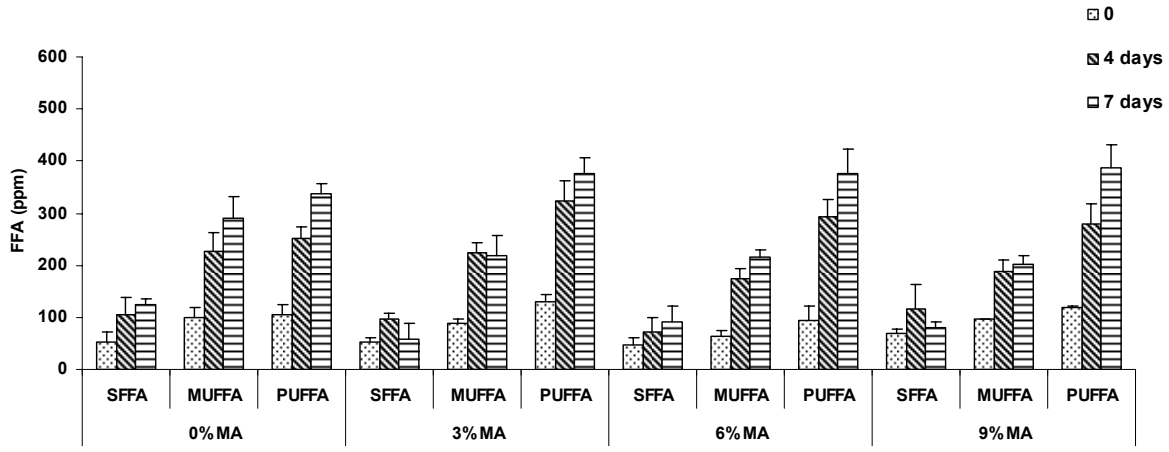


Figure III-3c: Aggregates of free fatty acids (FFA) released during storage in Rabbit minced meat obtained from animals fed with different diets and stored at 4°C under Vacuum (VAC).

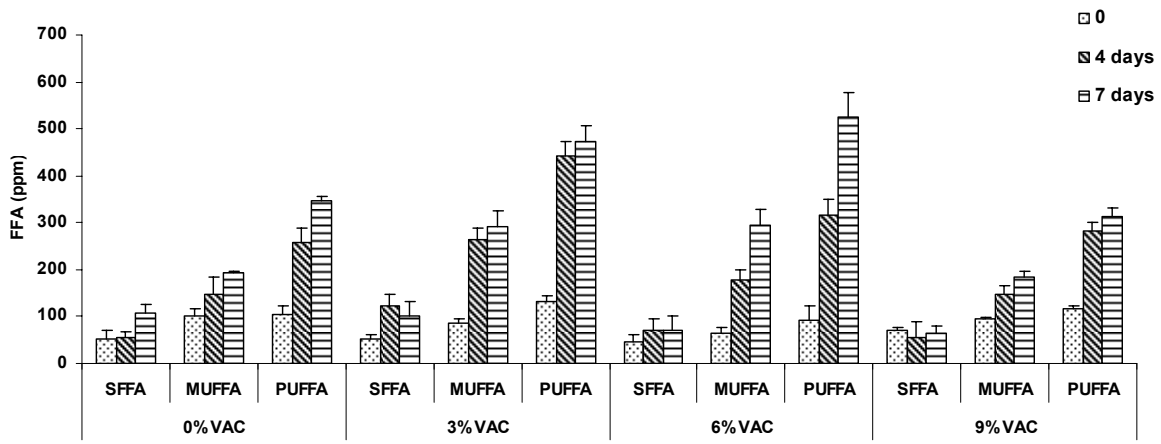
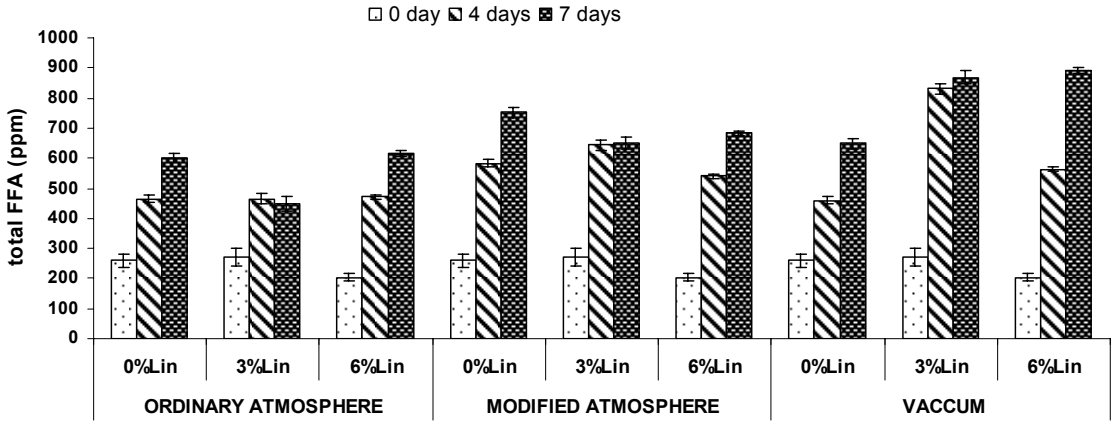


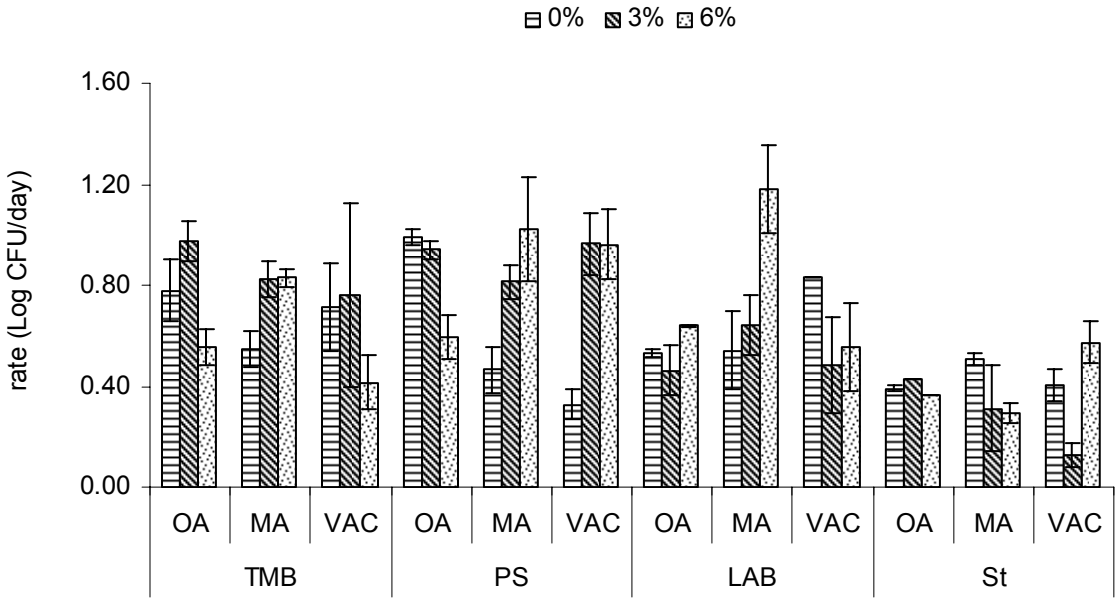
Figure III-4 Total free fatty acids released at 0, 4 and 7 days of storage in Rabbit minced meat obtained from animals with different diets and stored at 4°C under OA, MA and VAC



III-3-1 Microbiological analyses

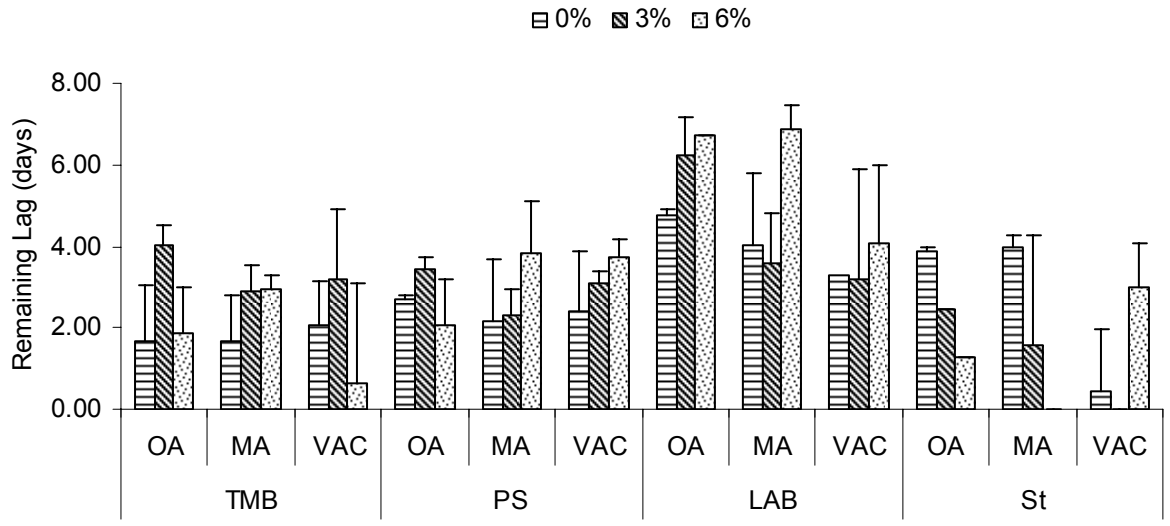
Microbiological analyses were performed over time on the various samples to assess the influence of fat composition and storage atmosphere on the different bacterial groups growth dynamics and product shelf-life. Only the control, 3% and 6% samples were taken into consideration because the 9% linseed supplementation showed to induce animals feed intake limitations and consequently low rabbit mass growth. In figure III-5 the growth rates obtained by fitting the microbial groups growth curves from the different meat product types with the Baranyi and Robert (1994) models are reported. Total mesophylic (TMB) and Psychrotrophic (PS) bacteria growth rates decreased with the increase of linseed percentage when the samples were stored in ordinary atmosphere, while lactic acid bacteria and *Staphylococcus* spp. (St) growth rates in OA did not show any difference associated to the diet. Apart from St, all the other bacterial groups had an increasing trend of their growth rates when the samples with increasing levels of PUFA in the meat were stored in MA. The enrichment in PUFA did not favour the growth rates of TMB and LAB when stored under vacuum as on the contrary it was the case for PS. In general St had the lowest rates in all conditions with respect to the other bacterial groups.

Figure III-5: Growth rate and estimation standard errors of the different spoilage microrganism as function of diet linseed and storage atmosphere.



The Lag parameter estimated by fitting the growth curves with the Baranyi model can be regarded as the remaining lag time, because the different microbial groups in theory start the adaptation in the product already after slaughter. In Figure III-6, showing the remaining lag times of the different bacterial groups in the various samples stored in OA, MA and VAC, no significant differences can be observed between the lag times of TMB, PS and St, while lactic acid bacteria were those that showed the highest lag values, especially in samples packed in OA and MA.

Figure III-6: Remaining lag times and estimation standard errors of the different spoilage groups as a function of diet linseed and storage atmosphere.



In order to evaluate the shelf-life of the different product types, the times to attain a level of 7 Log CFU/g of total mesophylic bacteria and Psychrotrophic bacteria were extrapolated. In Figures III-7a and III-7b the times to reach the cell load of 7 Log CFU/g in the different samples are reported. Based on TMB, the shelf-lives of the samples were longer in VAC, followed by OA storage, while the linseed percentage in the diet did not influence the TMB lag times. The times for psychotropic bacteria to reach 7 Log CFU/g were very high in the vacuum storage with respect to the other storage conditions. In particular, the PS times to 7 Log CFU/g in the control

were 10 days, 8.33 days and 5.38 days for VAC, MA and OA, respectively. In OA, the time to 7 Log CFU/g of PS decreased with the increase of linseed percentage, while it showed an opposite trend in AM and VAC packed samples.

Figure III-7a: Times for total mesophylic bacteria to reach the level of 7 Log CFU/g in the various meat product types.

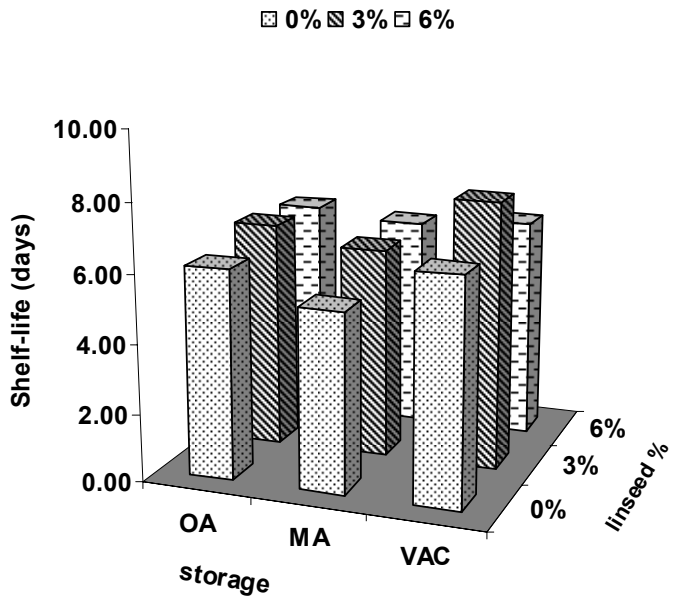
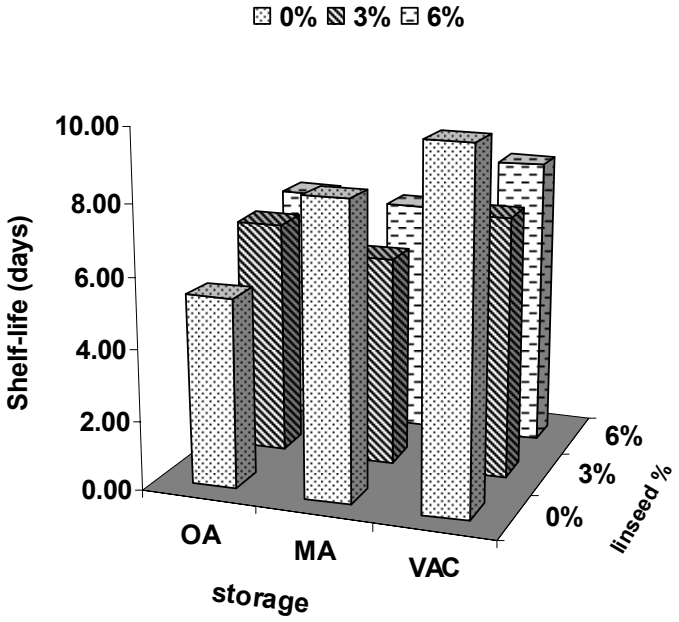


Figure III-7b: Times for total psychrotrophic bacteria to reach the level of 7 Log CFU/g in the various samples meat product types.



III-3-2 Thiobarbituric reactive substances analyses

In order to assess the level of oxidation of the different samples as a function of the diet and storage atmosphere, the thiobarbituric reactive substances analyses were performed at 0 and 7 days of storage. In OA packed samples, the oxidation level increased with the linseed percentage in the diet, while in modified atmosphere and vacuum an opposite trend was observed (figure III-8a). The oxidation phenomenon in refrigerated products can be a consequence of chemical and microbial enzymatic activities. In this perspective, the incidence of the microbial activity in the product oxidation was assessed by preventing the growth of the major microbial spoilage groups during storage of some samples within each product type. For this reason, minced meat from rabbit meat originating from the different diets were added with nisin and Chloranphenicol at 1000ppm each and stored in OA and VAC. The oxidation levels of these samples were measured after 7 days of storage. In Figure III-8b it can be observed that in all the meat product types, with the only exception of control one in OA, in the absence of the majority of the spoilage flora, the oxidation levels were lower than in the samples without antimicrobials.

Figure III-8a: Oxidation levels expressed as malondialdehyde content in the different samples stored in OA, MA, VAC.

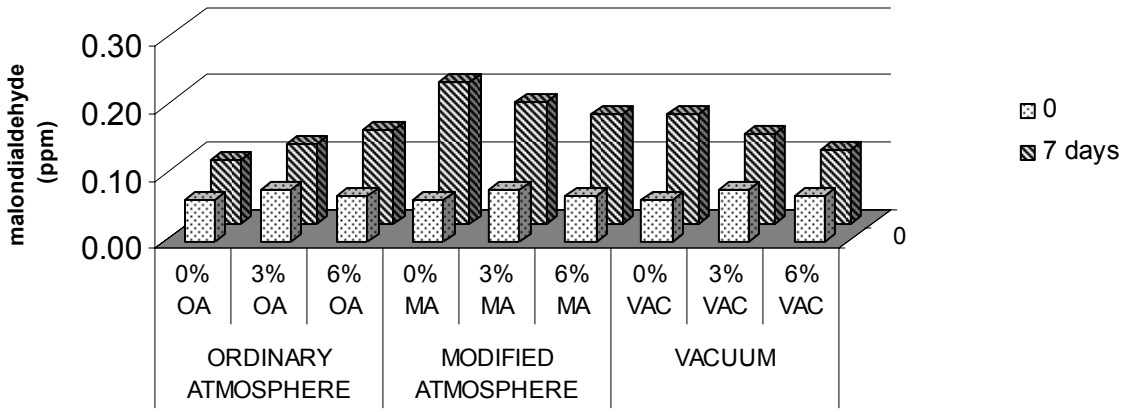
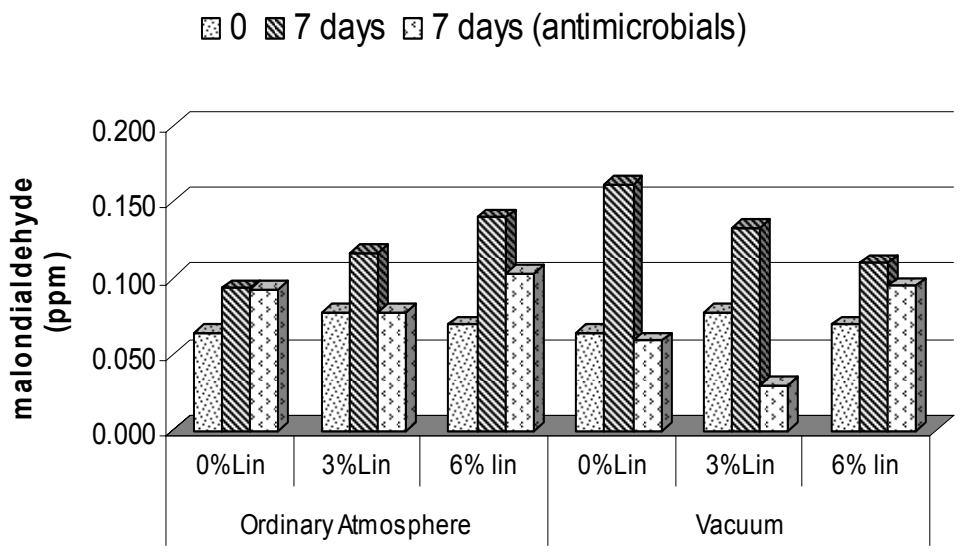


Figure III-8b: Oxidation levels expressed as malondialdehyde content of samples with and without antimicrobials, at 0 and 7 days of storage in OA and VAC.



III-3-3 Head space volatile compounds

In Table III-6 the volatile molecules detected in a head space of the different samples at 0, 7 and 11 days of storage, detected using the solid phase micro extraction combined with a GC-Mass device, are reported. The compounds levels are expressed as chromatographic area divided by 1000 for the sake of simplicity of vision. Detailed comments of the results have been done using figures extracted from this table. the head space volatile molecules analyses revealed more than 30 compounds, not always present in all the meat product types. Some of the compounds like the terpenes originates principally from spices, while others like acetic acid, isoamylic alcohol, 1,3-butandiol, 2,3-butandiol, acetoin are usually correlated to microbial growth. Figure III-9a and III-9b represent respectively the levels of 1,3-butandiol and 2,3-butandiol in the samples head space during the storage a 4°C in the three different atmospheres, namely ordinary atmosphere (OA), modified atmosphere (MA) and vacuum (VAC). The two compounds were

present only at the 11th day of storage, hence after the shelf life period. In particular 1,3-butandiol was produced at higher levels under modified atmosphere, but it was also present in the other atmosphere conditions. On the contrary, 2,3-butandiol was produced in the same proportions under both OA and MA conditions, while it was absent in the head space of the samples stored under vacuum.

Table III-6: Head space volatile compounds, expressed as chromatographic peak area, during storage of rabbit minced meat from animals fed with different diets and stored in OA, MA and VAC.

Compounds	0%T0	0%T7AO	0%T11AO	3%T0	3%T7AO	3%T11AO	6%T0	6%T7AO	6%T11AO	0%T0	0%T7MA	0%T11MA	3%T0	3%T7MA	3%T11MA	6%T0	6%T7MA	6%T11MA	0%T0	0%T7VA	0%T11VA	3%T0	3%T7VA	3%T11VA	6%T0	6%T7VA	6%T11VA
acids																											
acetic acid			2.32			1.36		0.99	17.57		4.97	19.97			2.61		16.26	14.12		1.81	3.31		1.63	7.34		2.97	28.92
alcohol																											
ethanol			14.79		3.82	12.53		1.93	12.96			9.78		1.27	5.84		7.89	21.34		17.77	45.66		4.55	34.67		12.23	32.35
isoamylic alcohol				17.82	65.50				0.89				17.82	2.59	72.74							17.82					33.63
4-methyl-1pentanol																											
1-heptanol																											
2-ethyl-1hexanol		21.58	9.69		16.79	27.98		57.69	13.12			2.31								2.78	16.42						0.76
1,3-butandiol			18.93			8.12			2.59			42.48			28.45			11.51		1.30	6.80			3.86		1.70	22.29
2,3-butandiol			34.19			32.91			41.39			27.93			3.47		2.93	21.47									
2-nitro-1-phenyl-1,3-propandiol	8.38			1.79	5.29	5.52	2.89	1.91	4.16	8.38	8.55	13.56	1.79	6.17	7.46	2.89	9.51	12.19	8.38	1.75		1.79	4.24	3.15	2.89	13.18	5.48
3 nonin-1-ol	3.46	6.20	44.69	5.59	35.27	18.14	17.96	1.72	69.31	3.46	8.33	1.48	5.59	2.63	3.45	17.96	1.77	57.25	3.46	8.27	11.83	5.59	2.55	74.52	17.96	31.90	51.36
furfurylalcohol	18.73	2.25	4.85	6.66	4.71	22.73	34.72	1.66	7.86	18.73	3.98	18.56	6.66		7.23	34.72		7.19	18.73	6.49	32.20	6.66	3.88	18.49	34.72	2.49	9.21
aldehydes																											
butandial						1.65														9.96	13.28		1.29	12.67		12.23	14.78
hexanal												4.33												4.90			
esters																											
ethylacetate		48.37	45.60		53.94	34.14		36.95	18.97					0.94			1.76			1.10			2.95				0.96
ethenylacetate		0.69	19.33			14.94			9.43		1.97	18.75		2.16	11.76		1.12	36.34									4.45
methyloctanoate																											
methyldecanoate												1.34															
Hydrocarbons																											
2,2,4,6,6-pentamethylheptane		88.29	7.62		5.51	8.12		11.94			22.57	3.76		8.97	0.88		13.89	3.39			6.32		1.29			4.43	
ethylbenzene																											
1,2,dimetylbenzene			1.49	1.78		1.23						2.79	1.78								2.25	1.78		1.81			
triisobutylene	27.49									27.49									27.49								
2,2,4,6,6pentanethyl 3heptene				45.68			63.49						45.68			63.49						45.68			63.49		
trans 2,4nonadiene	55.70						75.53			55.70						75.53			55.70						75.53		
1,2 dipenthyl cyclopropene				57.22									57.22									57.22					
1,3-di-tert-butylbenzene											1.65	3.40		6.33	4.85		12.81	4.76									
1-etil-2-metilciclopentene						15.43																					
terpenes																											
paracimene		27.37	1.68		14.77	53.27		18.63	28.44					1.14				1.30					0.99				
gamma terpinene		3.96	1.14		1.94	15.63		1.83	4.93																		
delta-3-carene	5.18	2.44	2.18	18.12	4.18	8.00	4.56			5.18	4.59	6.65	18.12	9.59		4.56	19.34		5.18	17.11		18.12	3.97		4.56	13.64	6.67
beta ocimene		1.82	1.52					25.58			9.37				9.49			12.42					3.45	14.58			
limonene	1.89	124.83	156.97			165.12	11.69	96.11	48.48	1.89	17.33	27.61				11.69	26.97	75.82	1.89	2.89			13.52	22.75	11.69	21.76	
trans-caryophylene	43.76	25.66	29.39	47.00	46.81		78.84	98.93	99.40	43.76	3.87	65.24	47.00	34.19	42.22	78.84	45.84	76.74	43.76	44.98	67.77	47.00	41.76	71.90	78.84	37.89	48.98
Ketones																											
acetone	17.65	5.68	13.74	5.52	1.63	14.11	14.27	11.95	25.37	17.65	32.51	26.29	5.52	25.42	17.44	14.27	36.18	26.39	17.65	14.56	2.69	5.52	9.14	18.76	14.27	11.79	7.10
2-butanone	1.68	0.95		0.91	6.53		1.52			1.68	9.34	3.94	0.91	4.74	5.97	1.52	1.14	4.38	1.68	11.34	0.91	0.91	7.96	3.53	1.52	5.78	4.62
acetoine		81.66	285.60		11.23	37.87		57.79	524.67		117.77	288.61		134.19	349.92		148.53	439.93		24.33	29.14		8.93	3.68		19.70	82.42
furanes																											
2-pentylfurane			2.66			11.81			3.943																		

Figure III-9a: Presence of 1,3-butandiol in the head space of samples obtained from rabbit meat fed with diets based on different linseed content and stored in under diverse atmospheres at 4°C.

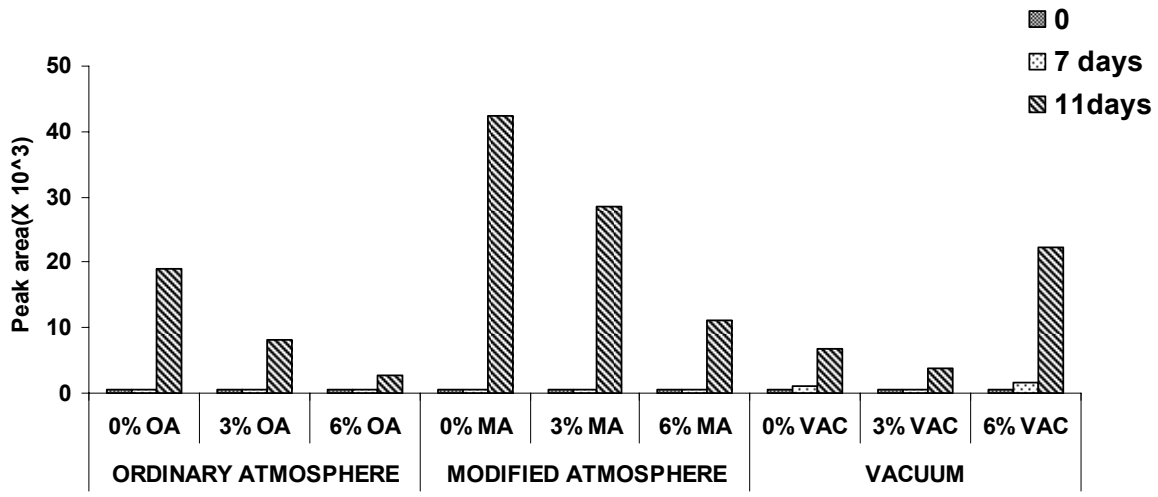
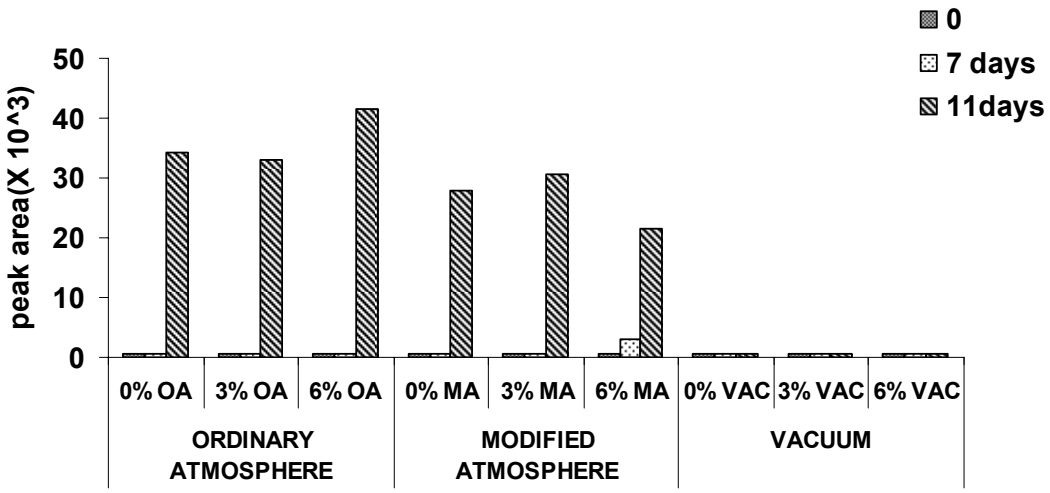
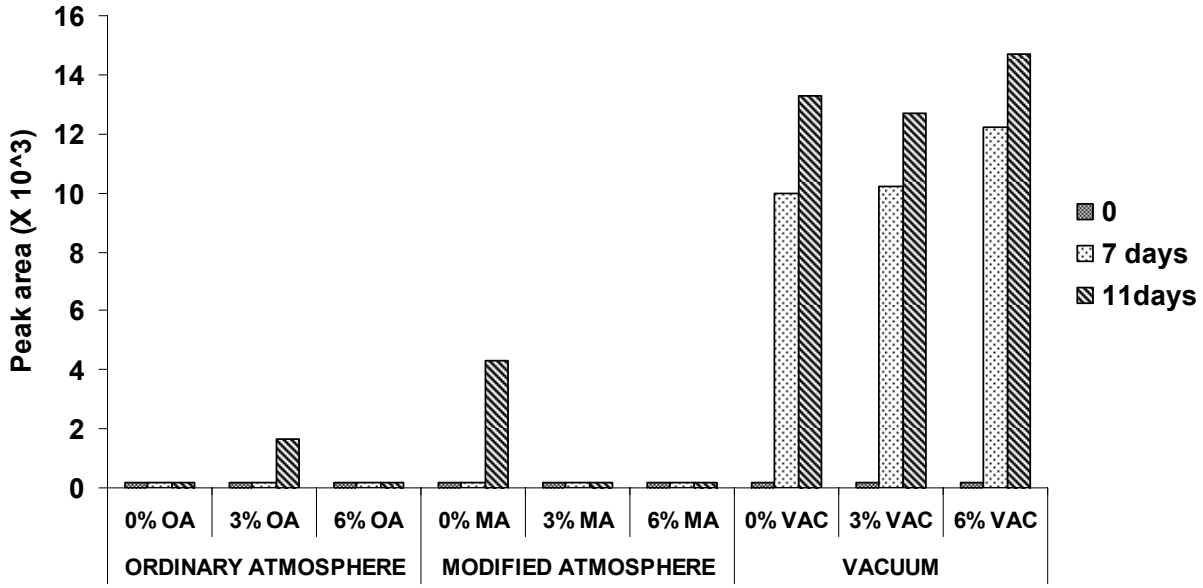


Figure III-9b: Presence of 2,3-butandiol in the head space of samples obtained from rabbit meat fed with diets based on different linseed content and stored in under diverse atmospheres at 4°C.



Hexanal was present only sporadically, while the other aldehyde revealed, i.e. butandial, was detected at the end of shelf-life period and over as indicated in figure III-10. Moreover, the latter compound was present particularly in the head space of samples stored under vacuum.

Figure III-10: Presence of butandial in the head space of samples obtained from rabbit meat fed with diets based on different linseed content and stored in under diverse atmospheres at 4°C.



Acetoin, whose presence is generally correlated with meat spoilage, was present only in trace in the head space of samples stored under vacuum, indicating a low rate of spoilage in samples under his condition. Moreover, the increase of linseed percentage was followed by that of acetoin, especially after 11 days of storage (Figure III-11).

Also the total ester compounds were present only in traces in the samples stored under vacuum, while they were detected in high quantities even at the 7th day of storage in OA; theses compounds were found only after the shelf-life period in MA packed samples (figure III-12). Moreover, ethanol was particularly produced in high quantities only in the head space of samples at the 7th and 11th day of storage under vacuum (figure II-13).

Figure III-11: Presence of acetoin in the head space of samples obtained from rabbit meat fed with diets based on different linseed content and stored in under diverse atmospheres at 4°C.

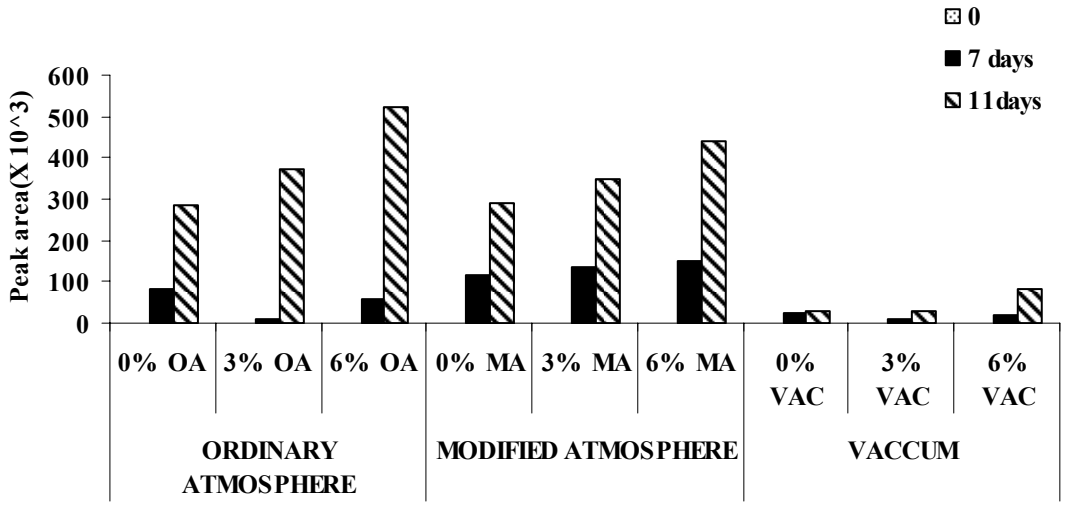


Figure III-12: Presence of total esters in the head space of samples obtained from rabbit meat fed with diets based on different linseed content and stored in under diverse atmospheres at 4°C.

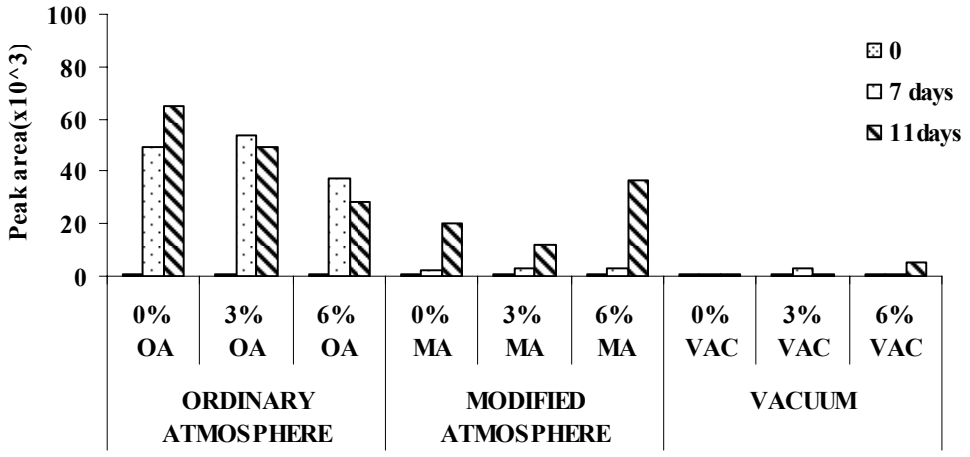
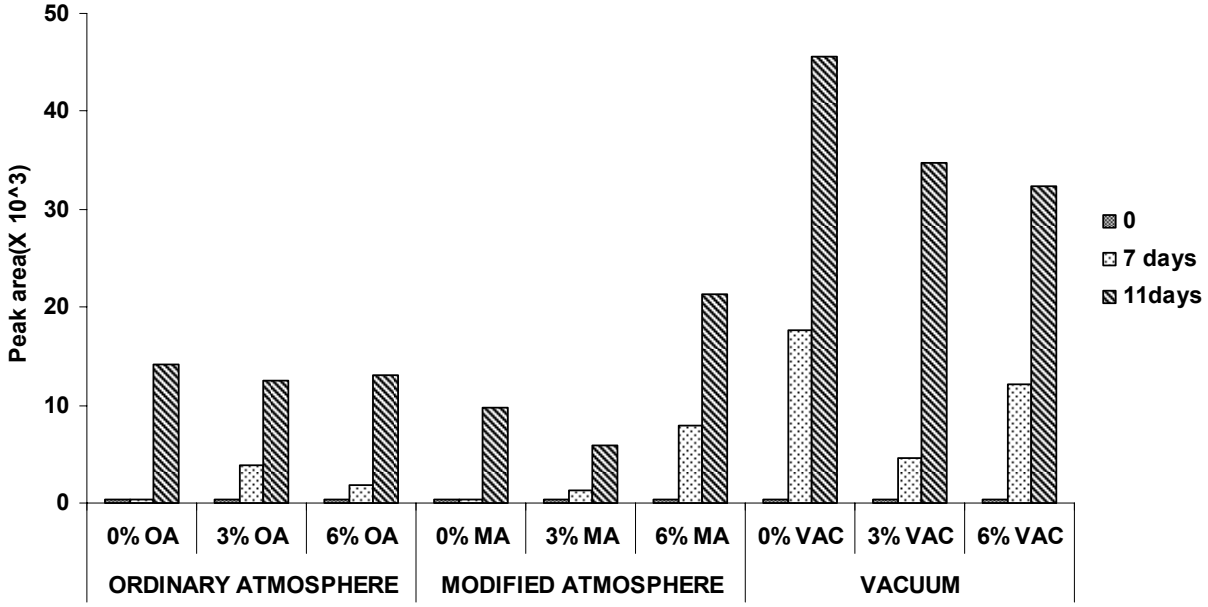


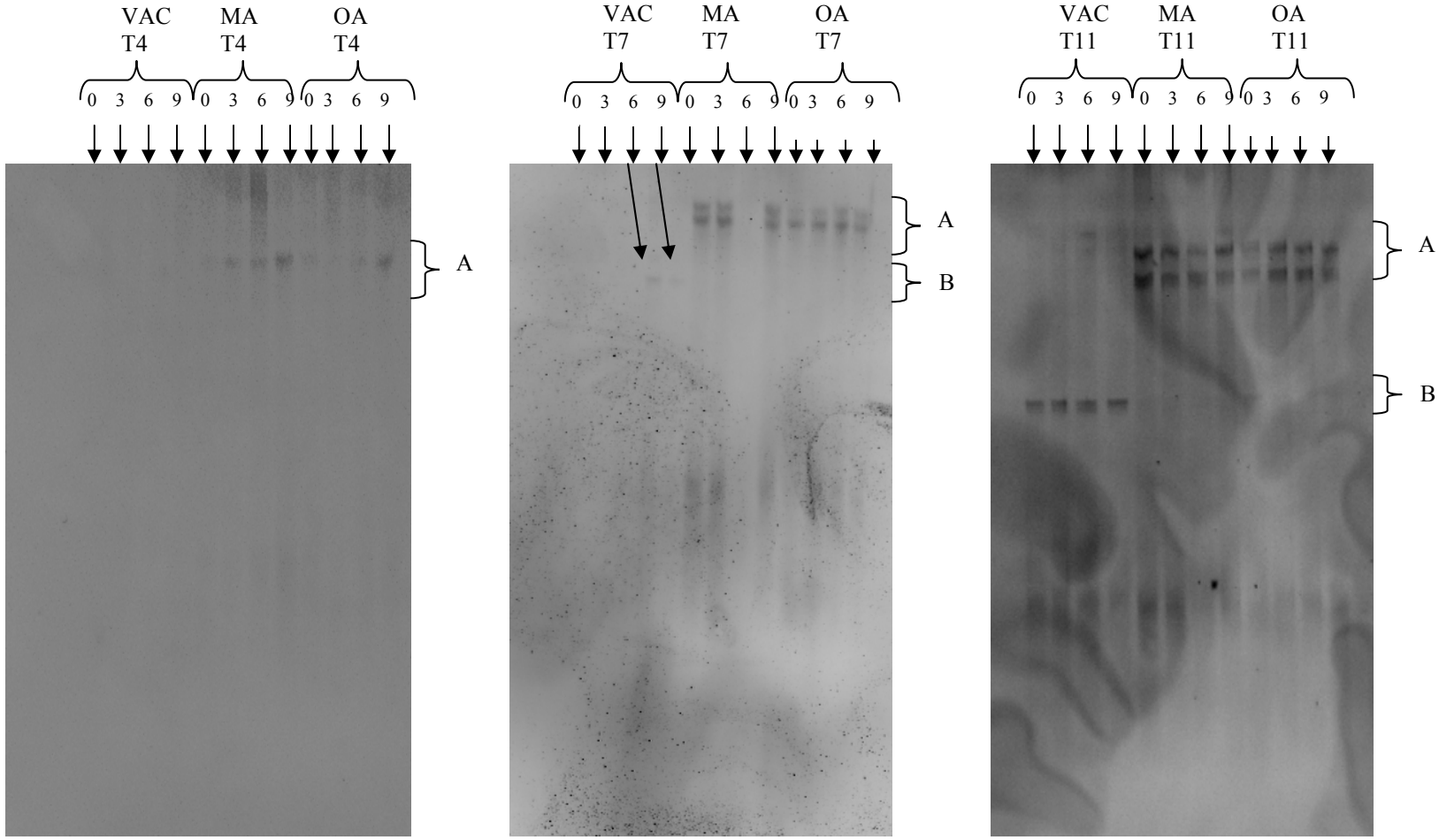
Figure III-13: Presence of ethanol in the head space of samples obtained from rabbit meat fed with diets based on different linseed content and stored in under diverse atmospheres at 4°C.



III-3-4 Direct analysis of meat products by DGGE

Total DNA was extracted from each meat product type and they were used in PCR to obtain the V1 region product that was analyzed by DGGE. According to Cocolin et al. (2001) such primers were considered suitable for obtaining good differentiation among. *Lactobacillus, Staphylococcus* and *Kokuria.* In figure III-14, the patters of the PCR products obtained with the primer pair P1-P2 for the amplification of DNA related to products sampled at day 4 (T4), 7 (T7) and 11 (T11) are reported. The comparison of the DGGE evidenced different profiles in relation to the packaging conditions. In fact at day 4 two bands (band A) were detected in OA and MA packed samples, while they were not present in VAC products. Such bands remained throughout the whole monitoring period showing increasing intensities. After 7 days of storage a new band (band B) appeared in the gel for the VAC samples, and namely for 6 and 9% VAC; moreover, this band was obtained after the 11^{th} day for all the VAC samples with high intensity.

Figure III-14: DGGE profiles of the DNA amplicons obtained directly from the rabbit minced meat after 4, 7 and 11 days of storage. Meat obtained from rabbits fed with 0, 3, 6 and 9% linseed in their diets were stored under vacuum (VAC), modified atmosphere (MA) and ordinary atmosphere (OA)



III-3-5 ANCOVA analyses of the influence of diet, storage atmosphere and time of storage on the variability of key dependent parameters measured

The ANCOVA analyses was performed using diet and storage atmosphere as categorical variables, and time as continuous one. In the tables III-7a, III-7b, III-7d, III-7c and III-7d, the contribution of the three factors on the variability of the parameters measured are reported. The influence time of storage on the parameters when assessed, was always significant except in the case of the release of 1,3-butandiol and 2,3-butandiol (table III-7c). On the contrary, other volatiles compounds released in the samples head space (acetoin, total esters, butandial and ethanol), were significantly influenced by both storage time and atmosphere (table III-7c). The atmosphere of storage and the diets did not significantly affect the growth over time of TMB an LAB (table III-7a) while, when considering the growth parameters, the remaining lag time of lactic acid bacteria was influenced by diet ($p< 0.05$)and storage atmosphere (table III-7d). On the other hand, there was a combined effect of diet and storage atmosphere on the psychrotrophic bacteria (PS) and staphylococci microbial counts over time ($p< 0.05$), while the single factors were not significantly influent (table III-7a). As expected, the linseed percentage in the diets significantly influenced the variability of the meat fatty acids aggregates (i.e. tot SFA, tot MUFA and tot PUFA). Regarding the released free fatty acid, the time of storage was obviously a significant factor in the aggregate variability ($p< 0.001$), while only the total polyunsaturated free fatty acids (tot PUFFA) were influenced by the atmosphere of storage (table III-7b). The influence of the diet and storage conditions on the TBARS values at day 0 and 7 of storage was also assessed. The linseed percentage of the diet did not influence the oxidation level during storage, while atmosphere of storage significantly ($p<0.05$) influenced theses values at 7^{th} day of storage (table III-7d).

Table III-7a Influence of the experimental factors on the growth counts variability of TMB, PS, LAB and St

	Factors					Model vs residues SS test	
	Time of storage	Atm	% linseed	Atm x % linseed	error	F	P
TMB	**116.52****	0.86	0.53	1.48	14.39	**29.60**	**0.0000**
PS	**109.55****	1.85	0.79	**2.75***	21.77	**18.50**	**0.0000**
LAB	**47.55****	5.66	0.13	0.66	19.79	**10.64**	**0.0000**
St	**34.36****	0.25	0.41	**3.44***	8.01	**18.35**	**0.0000**

Atm. (atmosphere of storage); * (significant at 95%); ** (significant at 99.99%)

Table III-7b Influence of the experimental factors on the variability of aggregates of free fatty acids released over time.

	Factors					model vs residues SS test	
	Time of storage	Atm	% linseed	Atm x % linseed	error	F	P
tot SFFA	**6893.6****	1021.7	1231.2	2058.0	5715.1	**3.70**	**0.00974**
tot MUFFA	**99011.2****	7850.5	2070.7	12555.0	19021.7	**12.06**	**0.00001**
tot PUFFA	**303020.6****	**31594.2***	16545.2	8251.4	51425.2	**13.20**	**0.00001**

Atm. (atmosphere of storage); * (significant at 95%); ** (significant at 99.99%)
tot SFFA (total saturated free fatty acids); tot MUFFA (total monounsaturated free fatty acids)
tot PUFFA (total polyunsaturated free fatty acids)

Table III-7c Influence of the experimental factors on the variability of selected volatile compounds released in the samples head space over time

	Factors					model vs residues SS test	
	Time	Atm	% linseed	Atm x % linseed	error	F	P
acetoino	**284747.5****	**109651.8***	12611.5	3330.8	221991.7	**3.49**	**0.013**
esters	**8537.66****	**7109.4***	2246.48	2867.39	10869.59	**3.61**	**0.011**
1,3 butandiol	931.1	182.94	67.67	218.9	1507.34	1.75	0.152
2,3 butandiol	1587.66	709.35	0.71	19.74	2439.78	1.79	0.143
butandial	**123.62****	**357.89***	0.629	7.343	194.32	**4.76**	**0.003**
ethanol	**1757.17****	**760.32***	47.8	157.135	1156.84	**4.44**	**0.004**

Atm. (atmosphere of storage); * (significant at 95%); ** (significant at 99.99%)

Table III-7c Influence of the experimental factors on the variability of microbial growth parameters, Tbars and meat lipid composition aggregates.

	Factors			model vs residus SS test	
	% linseed	Atm	error	F	P
rate CMT	0.010	0.032	0.199	0.35	0.79
rate PS	0.106	0.014	0.417	0.48	0.71
rate LAB	0.037	0.094	0.285	0.77	0.56
Lag CMT	0.002	0.635	7.688	0.14	0.93
Lag PS	0.97	0.23	2.61	0.77	0.56
Lag LAB	**5.29***	**8.46***	3.61	**6.36**	**0.04**
Tbars T0	0.00004	0.00000	0.00026	0.25	0.86
Tbars T7	0.00047	**0.00756***	0.00311	**4.30**	**0.08**
tot SFA	**31.88***	0.00	17.43	3.05	0.13
tot MUFA	**16.31***	0.00	6.43	**4.22**	**0.08**
tot PUFA	**90.72***	0.00	46.42	3.26	0.12

Atm. (atmosphere of storage); * (significant at 95%)

tot SFA (total saturated fatty acids); tot MUFA (total monounsaturated fatty acids)
tot PUFA (total polyunsaturated fatty acids)

III-4 Discussion

In this investigation the influence of polyunsaturated fatty acids supplementation of rabbit fodder on the microbial and chemical parameters of minced meat stored under different atmospheres was assessed. The fatty acid synthesis in animal occurs principally in the muscles, adipocytes and hepatic tissues. Rabbit distinguish themselves form birds (whose lipogenesis is principally hepatic) and also from pigs and ruminants (with principally adipocyte tissues lipogenesis) because their *de novo* fatty acid synthesis take place both in hepatic and adipocyte tissues (Gondret, 1999). The fat contain of the minced meat was higher in the controlled samples with respect to those originating from rabbit fed with PUFA enriched diet. It has been demonstrated that the supplementation with a PUFA source of rabbit diet reduces the lipogenic enzymes activity in the liver and that muscle fatty acids content depends more on what is diffused in the blood than on lipid synthesis (Gondret et al.,

1998b). The fact that lipogenesis *de novo* does not contribute much on the muscle fatty acid is also confirmed by the significant influence of the diet linseed percentage on the variability of the rabbit minced meat fat composition.

Regarding the free fatty acids, their release during storage in minced meat can be related to endogenous and bacterial enzymatic activities. Atmosphere of storage in our experiment also influenced the PUFFA release, indicating the involvement of microbial growth, although endogenous enzymes sharply increase the amount of free fatty acids, especially that of PUFFA during refrigerated storage of rabbit meat (Alsanier et al., 2000). Moreover the same Authors also observed that the contribution of phospholipids to the FFA fraction was twice that of triacylglycerols in the glycolitic muscles, whereas it was similar or lower to that of triacylglycerols in the oxidative muscles. Lipases as well as proteinase are generally produced by *Pseudomonas*, preferably at low temperatures (Witter et al., 1966; Olson and Nottingham, 1980). Other bacterial populations as *Micrococacceae*, yeast and lactobacilli also possess esterases and lipases that may contribute to the FFAs release (Casalburi et al., 2006; Montel et al., 1996; Lanciotti et al., 2005). Although the PUFFA release depended on the atmosphere of storage and linseed percentage of the diet, oxidation was significantly affected only by the former factor. This can suggest a bacterial implication in the fatty acid oxidation or in its prevention, but also a correlation with the high oxygen content of the protective atmosphere. In our experiment, the implication of oxygen and light in the oxidation processes was very low as demonstrated by the comparison between oxidation levels in samples with and without antimicrobial compounds (figure III-8b). On the other hand, under the vacuum storage, a more complex mechanism can be responsible for lipid oxidation process. Many Authors have described the relation between lipid oxidation and oxidation of meat pigments (Faustman et al., 1989; Gatellier et al., 1992; Renerre and La badie, 1993) but less research have been done on rabbit, chicken and turkey meats. In fact, low oxygen pressure atmosphere favour the oxymyoglobin oxidation that results in the production of three chemical species; metmyoglobin, l'H_2O_2 and O_2^{-0} (Renerre, 2000). Interaction of metmyoglobin and H_2O_2 generates activated metmyoglobin that Davies (1990) and Giulivi and Cadenas (1993) suggested to be a ferrylmyoglobin radical P^{+0}-Fe^{IV}=0. This very unstable radical decays rapidly to form a peroxyl radical which is capable of initiating lipid oxidation in many biological systems (Chan et al., 1997; Kanner and Harel, 1985; Witting et al., 1999). On the other hand, Chan et al. (1997) demonstrated that secondary lipid oxidation products were pro-oxidative towards oxymyoglobin oxidation, while Gotoh and Shikama, (1976) demonstrated by an *in vitro* experiment the role of Superoxide

dismustase (SOD) and Catalase in the oxymyoglobin oxidation. For example, *Pseudomonas* spp. has the catalase enzyme, while lactic acid bacteria, which are catalase negative, produce H_2O_2 that can form, an active complex with metmyoglobin as stated before.

The microbial flora of the different samples was dominated by psychrotrophic and lactic acid bacteria irrespective of the atmosphere of storage and diet. Among the different bacteria groups assessed, only the cell loads over time of PS and St were significantly affected by linseed percentage of the diets and storage atmosphere. The high lipolysis activity of these two bacterial groups as discussed previously also explain the effect of atmosphere of storage on the PUFFA release and Tbars. The overall shelf-life of the products did not show any significant difference with respect to the linseed percentage of the diet and the atmosphere of storage when total mesophylic bacteria was taken into account. On the contrary, the shelf-life based on the growth of psychrotrophic bacteria increased in relation the atmosphere of storage in the following order OA>MA>VAC. This indicates that the composition of the microflora probably was affected by the type of storage and maybe by the fatty acid composition. In fact atmosphere of storage is known to influence the growth of bacteria and the absence of this influence can be interpreted as an indication of different environment or population composition. Another evidence of the different microbial composition and/or metabolism according to the samples characteristics is given by the composition of the samples head space volatiles. 2,3-butandiol and 1,3-butandiol were not influenced by none of the factors, time of storage included. In particular 2,3-butandiol was found to be produced by milk natural microflora (Moio et al., 1993), denoting its correlation with aerobic conditions and lactic acid bacteria. In fact this compound was absent in samples stored under vacuum conditions. Moreover, acetoin, generally related to spoilage bacteria in meat (Intrapichet et al., 1990), and that also derives from the oxidation of 2,3-butandiol (Romano et al., 2002; Machielsen, 2006), was present only in traces quantities in VAC conditions. On the other hand, total esters were present only in traces in the head space of samples stored under vacuum. It is widely accepted that esters can be formed by the esterification of FFAs with alcohols, mainly ethanol (Nardi et al., 2002; Collins et al., 2003). Hence esterases of LAB can be defined as alcohol acyltransferases that catalyse the transfer of fatty acyl groups from glycerides to either water (hydrolysis) or alcohols (alcoholysis) in aqueous systems (Hollander et al., 2005). Regarding the presence of butandial in the head space, the fact that this aldehyde was detected mainly in the samples stored under vacuum and the absence of correlation with Tbars data exclude its connection with the oxidation process. In general, the head space volatiles analyses indicated a variability that was influenced by time and atmosphere of

storage and not by the meat fatty acid composition. Most of the volatile compounds produced and identified with SPME-GC-MS analysis in the samples can be associated to the presence of lactic acid bacteria. the composition the volatiles produced in samples in OA an MA were different with respect to that of products stored under VAC. In particular, butandial and ethanol were present in high quantity only under vacuum. Ethanol is a spoilage product produced in packaged meat by heterofermentative *Leuconostoc* specie and *Carnobacteria* spp (Dainty and Mackey, 1992). The low pH decrease between the 1st and 11th day of storage (data not reported) in the products under vacuum, is also an indication of the selective growth of different LAB genera or other species with respect to the other samples stored in OA and MA. According to Cocolin et al. (2001), the primer pair P1-P2 used in this analysis can permit the amplification of DNA related to LAB and *Micrococcaceae*. The staphylococci cell counts observed during the storage period, was very near to the general accepted sensibility of the PCR-DGGE analysis indicating that the Gram-positive group monitored by the PCR-DGGE analysis was principally the lactic acid bacteria. The different profiles obtained on samples from the 4th, 7th and 11th day of storage indicate a different composition of the LAB flora with regard to the atmosphere of storage. bands A could be presumably attributed to homofermentant lactobacili spreading region while band B to the heterofermentant lactobacili. However, a better identification would require the excision of the bands from acryl-amide gel, re-amplification and sequencing.

III-5 Conclusion

The complexity of the system given by fatty acid variability, the atmosphere of storage and microbial population can lead to interactions that can result in different keeping quality characteristics. The PUFFA release was dependent on both endogenous and microbial enzymes and can be selectively utilized by lactic acid psychrotrophic bacteria to overcome the difficulties of low temperature and modified atmosphere. On the other hand, it becomes more important to investigate in detail the interaction between free fatty acids and different bacteria in a more controlled system.

III-6 References chapter III

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CHAPTER IV

MINIMAL INHIBITORY CONCENTRATION AND PROBABILITY OF INHIBITION OF SELECTED FREE FATTY ACIDS ON *Staphylococcus aureus* IN IN-VITRO CONDITIONS

IV-1. Introduction

The high concern about lipid quality in food have brought animal producers to find means of enhancing the polyunsaturated fatty acid (PUFA) contain of animal origin. In particular, the inclusion in the animal diet, of lipid from marine or vegetal source reduces the ratio of n6/n3 PUFA. (Wiseman and Agunbiade, 1998; Wood et al., 2003; Nuernberget al 2005; Bryhni et al 2002; Otten et al., 1993). This modification in the lipid composition also affects the technological stability of products obtained from the meat of these animals. (Bryhni et al., 2002; Sado et al., 2005). During the refrigeration process of meat, there is in fact a release of free fatty acids (FFA) by means of endogenous or microbial enzymes, (Montel et al., 1996; Alasnier et al., 2000). Their presence can either have a positive impact like in the case of flavour development in most Mediterranean cured meat products (Chizzolini et al., 1998), or be negative when their oxidation leads to the production of volatile compounds with off-flavour (Ordoñez et al., 1999). Among compounds that can naturally be present in food, having antimicrobial activities like bacteriocins, weak acids and essential oil from spices (Lanciotti et al., 2003; Lanciotti et al., 2004; Belletti et al., 2004), FFAs are those which have got less attention in the past years. FFA antimicrobial activity can be important in meat products, as they are always available in quantities higher than other natural antimicrobials, due to enzymatic or bacterial activity. Hence knowing their antimicrobial potentialities, more information can be achieved on synergic activities of different natural antimicrobials in food. Since the pioneer studies on the antimicrobial properties of fatty acids and derivatives on bacterial species done by Kabara et al., 1972, other works have followed, with a common limiting aspect; the assessment of FFA antimicrobial activity under optimal environmental conditions of microbial growth, while in food these generally change, and in some cases may not favour the antibacterial activity. For example the pH of non defected pork meat range between 5.8 and 6.5 (Lebret, B. 2004). During transformation like in the case of salami, the temperature passes from 2°C of minced meat and

ingredient mixing, to 20 - 25°C of the fermentation phase, 16 - 22°C of the drying then 12 to 15°C of the final product storage. During the same production period, the product pH varies from 5.5-6.2 to 4.9-5.3 after the fermentation process, then raises again to 5.6-5.8 in the final product. In fresh sausage, the minced meat and ingredient are stored at 10°c after stuffing into casings for the night before storing them at 2-4°C for commercialization (Sado Kamdem et al., 2007). *Staphylococcus aureus* is one of the gram positive bacteria frequently responsible for food poisoning outbreaks in meat and meat product (Nel et al., 2004). Atanassova et al., (2001) obtained a 25.9% and 51.1% prevalence of *S. aureus* analyzing samples of raw pork and uncooked smoked ham, while Nel et al., (2004) analyzing samples of meat before packaging of South African slaughtering units observed that the levels of *S. aureus* ranged between $3.8x10^3$ and $2.42x10^5$ with and average of $1.72x10^5$, although the national limit is 100 cells/g and the infective level being 10^5 cells/g. the S. aureus cell load at the beginning of storage can easily go out of control if the good manufacturing practices are not well applied. The difficulty in assessing the level of this FFA antimicrobial activity in food is also related to the dynamic nature of the food as environment. Traditionally, the antimicrobial effect of a compound has been assessed by defining its minimal inhibition concentration (MIC) or its effect on the growth parameters of the target microrganism. This approach, usually performed in vitro, usually takes into consideration only few parameters of the food environment. To have an idea on the effective inhibition effect, it is important to evaluate combining different factors correlated to the food to be studied. The choice of all the parameters is obviously not possible, but those which can easily influence the compound antimicrobial activity or the microrganism resistance are more important than others (Stewart et al., 2002). Temperature for instance influence the solubility of FFAs (Maeda et al., 1996) while the pH is important for the concentration of the undissociated and dissociated fraction of the fatty acids (Le Marc et al., 2002). A part from the food parameter variability, the strain variability also introduce in the MIC determination a certain level of uncertainty. In fact, the cell variability within a strain population affects the lag time and detection time variability (Baranyi and Robert, 1994; Robinson et al., 2001; Baranyi, 2002; Métris et al., 2003; Pin and Baranyi, 2006). In this perspective, the MIC can be considered as a non stable value, which together with the other variables (food and microrganism), contribute to the probability of a microrganism being inactivated or not. So, when the bactericidal compound concentration, the food environment and the cell physiology contribute to the total inactivation of the bacterial population, we can speak of the "No growth" situation, while in case of partial inactivation or no inactivation, we can speak of growth. This binary outcome give way for the use of the logistic regression to

model the results of the microbial inactivation as function of different parameters. Logistic regression is a widely used statistical modelling technique, and is the technique of choice when the outcome of choice is dichotomous (Ross and Dalgaard, 2004). During the last decade, logistic regression models have been used in sevral publications (Ratkowsky and Ross, 1995; Jenkins et al., 2000; Gardini et al., 2000 Lanciotti et al., 2001; Le Marc et al., 2002). Because regression techniques do not exist for binary data, the regression equation is usually related to the log odds, or *logit*, of the outcome of interest. This has the effect of transforming the response variable from a binary response to one that extends from -∞ to +∞ reflecting the possible range of the predictor variables, and has desirable mathematical features also (Hosmer and Lemeshow, 1989). The logit function is the following

Logit $P = \log (P/(1-P)$

Where P is the probability of the outcome of interest.

Logit P is commonly described as a function Y of the explanatory variables. i.e logit $P = Y= b_0+b_1X_1+……+b_nX_n$. This equation can be arranged to: $1/(1+e^{-Y})=P$, or $e^Y/(1+e^Y)=P$ where $X_1…X_n$ are the explanatory variables and $b_1…..b_n$ are the respective coefficients. The use of this function can then be a useful tool to assess the probability of the antimicrobial activity of a compound in a given condition.

The aims of this experiment was i-the determination of the minimal inhibitory concentration (MIC) of selected fatty acids on *Staphylococcus aureus* in different conditions of pH, inoculum level, temperature and time of incubation. ii- to assess the probability of no growth of *S. aureus* in relation to selected free fatty acids, pH and temperature.

IV-2- Method set-up

IV-2-1 Influence of different ways of FFA dissolution methods and solvents on the activity of Lauric acid on S. aureus

Fatty acid and solvent were all purchased from Sigma-Aldrich, St. Louis, MO, USA. Lauric acid was tested at a concentration of 2.50 mM (Kabara et al., 1972). To enhance the dissolution, the acid was first dissolve in ethanol 96% or dimethyl sulfoxide (DMSO) and then added in broth in order to have 1% solvent in the final solution. Also mechanical dissolution was done by using ultrasound (Starsonic Liarre, Casal fiumanese; Bologna Italy) and ultraturax (DI 18 basic, Yellow line; Optolab s.n.c. Modena, Italy). In the case of ultrasound, a solubilizer and emulsifying non-ionic

surfactant brij58 (Sigma Aldrich), was also used to stabilize the emulsion. The experiment was performed in BHI pH 7.2 at 37°C and bacterial counts were prepared on plates of BHI plus 18g of Agar per litter of broth.

In figure IV-1a, the inactivation potentials of lauric acid 2.50mM added by means of different techniques in relation to the dissolution methods are shown. lauric acid affected both the *S. aureus* cell load recorded immediately after its supplementation and the maximum level attained in the stationary phase independently of the dissolution method adopted. A higher level of growth anyway was observed when brij58 was used to stabilize the micelles form by the use of ultrasound. A similar result was observed also when adding Brij58 to the fatty acid before the ultraturax homogenization (data not shown). Tow solvents (ethanol and DMSO) at a level of 1% of the BHI broth solution, and brij58 an anionic surfactant with no antimicrobials activity was tested (figure 1-b). The results indicated that Brij58 unlike DMSO inhibited the growth of *S. aureus*, reducing the cell load after 26h of incubation at 37°C of about a unite log CFU/ml. consequently, solvent dissolution (in particular ethanol) was chosen to increase the solubility of free fatty acids in the rest of the experiment.

figure IV-1a: Effect of different methods of enhancing Lauric acid solubility on its antimicrobial activity on *S. aureus* SR231

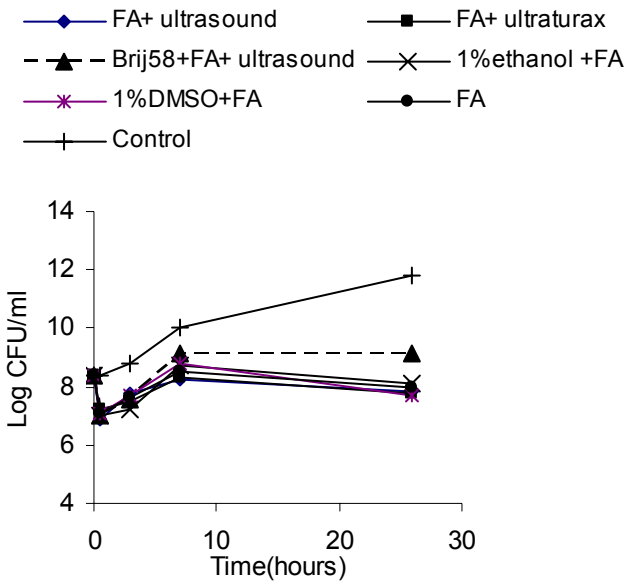
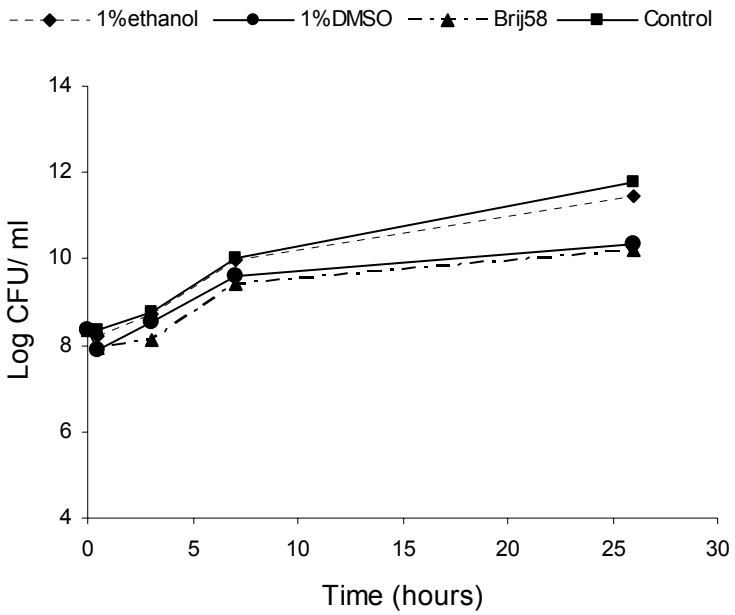


Figure IV-1b: Effect of ethanol, dimethyl solfoxide and Brij 58 on the growth of S. aureus SR231



IV-2-2 Solubility of selected fatty acids in 1% ethanol solution

Theoretically values of selected fatty acids in 1% ethanol solution were calculated based on the work of Maeda et al., (1996) using the UNIFAC method on water phase molecular fraction. The physical properties of some selected fatty acids obtained from literature are indicated in table IV-1. It can be observed that the difference of solubility between fatty acids decrease in an exponential way as the number of carbons of the aliphatic increases. Another interesting information is that short chain fatty acid pKas are quite similar while those of long chain fatty acids (saturated and unsaturated) are almost their double. A calculation of the theoretical values of the solubility of selected fatty acids in 1% (v/v) ethanol water phase solution by means of the UNIFAC method is presented in table IV-2. It can be seen that ethanol increases in a considerable way the solubility of the different fatty acids. In the case of Stearic acid, it was observed a lower calculated solubility respect to that of pure water available in literature. The prediction using the UNIFAC method, underestimated the solubility of Stearic acid. This can be explained by the highly nonideal behavior and polymorphism in solution of Stearic acid, as is soluble in both non polar and polar solvents and hence experimental data generally show strong deviations from predictions (Mirmehrabi and Rohani, 2004). The solubility increases with the reduction of the aliphatic chain and the increase of

double bonds and temperature. These data indicate that in some settings during our experiments in particular when using fatty acids with more than 12 carbons, the broth was in condition of FA saturation.

TableIV-1: Physical properties of selected fatty acids

Fatty acid	FW	formula	Pf°C	Solubility in H_2O at 20°C(g/100ml)	$LogP_{O/W}$[3] 25°C	pKa	K_p[2] (37°C) X 10^{-4}	K_p[2] (15°C) X 10^{-4}
propionic	74.08	C3:0	-21	Infinite	0.33[6]			
Butyric	88.11	C4:0	-5	Infinite	0.79[6]	4.82		
Valercic	102.1	C5:0	-34	4.97	1.39[6]			
Esanoic	116.1	C6:0	-3	0.968	1.92[6]	4.88		
Octanoic	144.2	C8:0	17	0.068	3.05[6]	4.89		
Decanoic	172.3	C10:0	32	0.015	3.92[4]	4.90		
Lauric	200.3	C12:0	44	0.0055	4.97[4]	4.90		
Tetradecanoic	228.4	C14:0	54	0.0020	6.02[4]			
Pentadecanoic	242.4	C15:0	53	0.0015	6.65[6]			
Esadecanoic	256.4	C16:0	63	0.00072	7.08[4]		67±2	240±30
Palmitoleic	254.4	C16:1			6.58[6]			
Stearic	284.4	C18:0	69/71[1]	0.00029	8.13[4]	10.15[1]		
Oleic	282.4	C18:1	13/14[1]		7.64[5]	9.85[1]	48±2	32±2
Elaidic	282.4	Trans C18.2	44 /45[1]		7.64[6]	9.95[1]		
Linoleic	280.4	C18:2	-5/-1[1]		7.05[5]	9.24[1]	14±2	5.6±0.4
α linolenic	278.4	C18:3	-11/-10[1]		6.46[6]	8.28[1]	4.9±0.5	1.5±0.2
Arrachidonic	304.5	C20:4	-50[1]		6.98[6]		14±2	5.2±0.5

[1]Kanicky and shah (2002). [2]partition coeficient in micelles of dimiristoleilphosphatidylcholine
[3]logarithm of partition coefficient in Octanol / water solution [4] Patton et. Al., (1984). [5]Sangster J. (1989).
[6]LogP predictor: ALOGPS 2.1 program (http://www.vcclab.org) Tetko, I.V. et., al 2005; Tetko, I.V. 2005

Table 2: Solubility of selected fatty acids in 1% ethanol solution

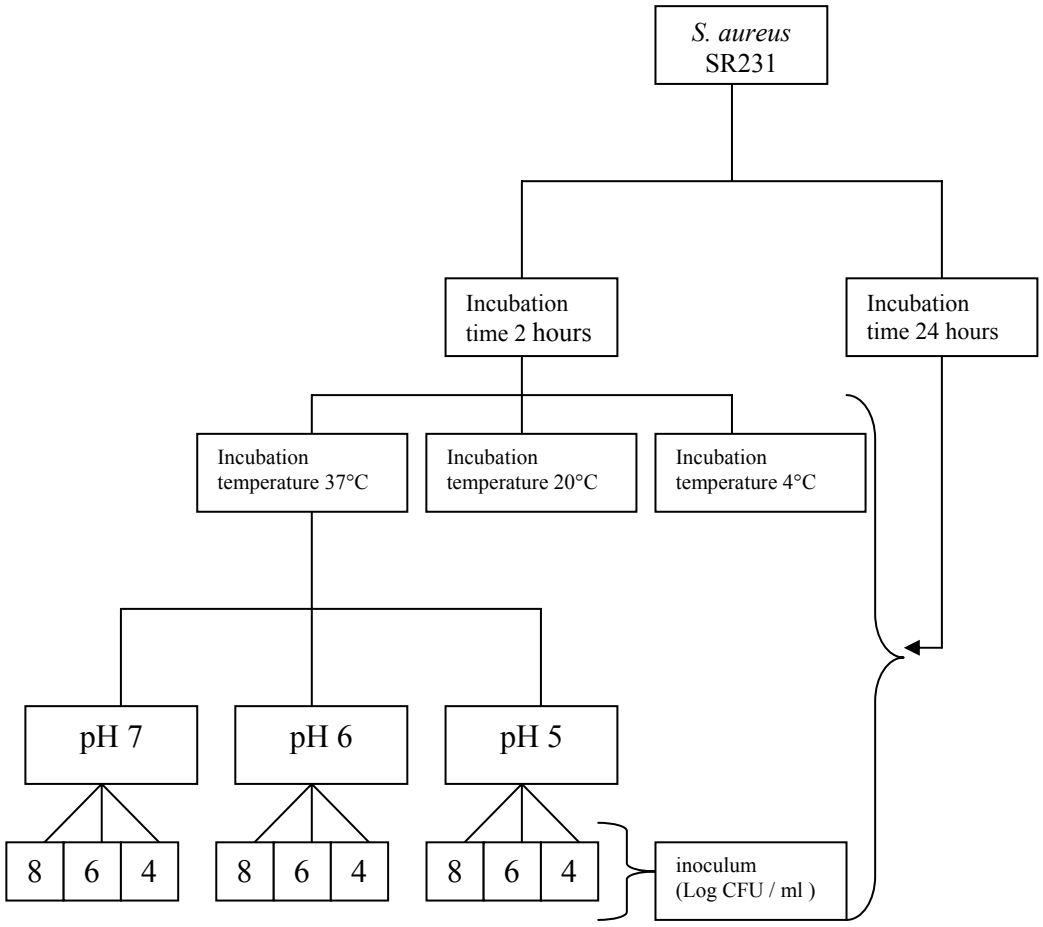
ppm FA in water 1% (v/v) ethanol water phase solution										
Temp.[K]	C8	C10	C12	C14	C16	C18	C16:1	C18:1	C18:2	C18:3
303	5827.19	5827.19	675.21	77.54	9.33	1.05	33.96	4.11	14.32	43.93
313	6631.83	6631.83	799.00	95.49	11.95	1.41	42.23	5.32	18.02	53.93
323	7500.22	7500.22	937.60	116.35	15.13	1.85	51.95	6.80	22.42	65.51

IV-3 Material and method

IV-3-1. Minimal inhibitory concentration (MIC) and no growth probability

The experimental plan (figure IV-2) was designed in order to simulate the conditions of temperature and pH observed in meat storage and fermented meat products. At the same time, the influence of the level of inoculum and the incubation period were assessed. Microtiter (Anicrin srl scorze (VE)-Italy) wells were used for the MIC determination, modifying the general technique commonly used. In practical, a series of dilution of the fatty acid was done in ethanol 96% in order to have regular concentrations intervals. Over testing concentrations of 1000ppm the interval was 50ppm, then between 100 and 1000ppm it was 25ppm, while under 100ppm it was 5ppm. For sample preparation, stock culture of *S. aureus* taken from –80°C in BHI broth plus 30% of glycerol (v/v) was sub-cultured in BHI both (pH 7.2) at 37°C for 24 hours, then passed on BHI-agar slopes which were incubated at 37°C for 24hours before storing it at 2°C for a monthly use. Before any analysis, the cells were passed on a BHI-agar plate and incubated at 37°C for 24h, and then one colony was sub-cultured in BHI broth (pH 7.2) for 24 h and 1ml transferred in 9ml of BHI broth of the same pH and incubated at the same temperature as the condition to be tested for 24h. BHI broth was used after sterilization at 121°C for 15 minute. These procedures were chosen to reduce the influence of cell adaptation during analysis on the MIC determination (in this case the cells were already adapted to the temperature of environment in which the FFA was tested). 99 µL prepared inoculum samples were then inoculated in Microtiter wells and 1 µL of the correspondent fatty acid concentration added in order to have a maximum of 1% ethanol in the final volume (This means that the FA solution was always a hundred times more concentrated than the value needed in the well and the highest concentration tested was 1500ppm for all FA). Incubation was done at the experimental plan condition for 2 and 24 hours. The experimental conditions tested for any incubation time and every FFA was 3 inoculum levels x 3 pH values x 3 incubation temperature x 32 FFA concentration = 864 After any incubation period, 10µL of the growth solution were inoculated in BHI-agar plate as spots (figure IV-3) and incubated at 37°C for 24 hours.

Figure IV-2: Experimental plan for the determination of the minimal inhibition concentration of selected fatty acids on *S. aureus* SR 231



IV-3-2: Data recording and analysis

Three types of results were considered: no growth, when no colony was present after 24hours incubation; more than 4 Log UFC cell reduction and no inhibition, when colony density were similar to the control. The MIC was determined as the lowest concentration that did not permit any growth after 24hours at 37°C of the 10µL of the growth solution on BHI-agar plate. Data of linolenic and Lauric acid were repeated twice while for other fatty acids it was done so only when some doubt occurred. For the no growth probability (NGp), data was recorded in a binary form, considering "1" as no growth and "0" as presence of colony. The data was then modelled using a logistic regression. A logistic-regression analysis was conducted on the raw data using Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy) in order to assess the probability of no growth during the

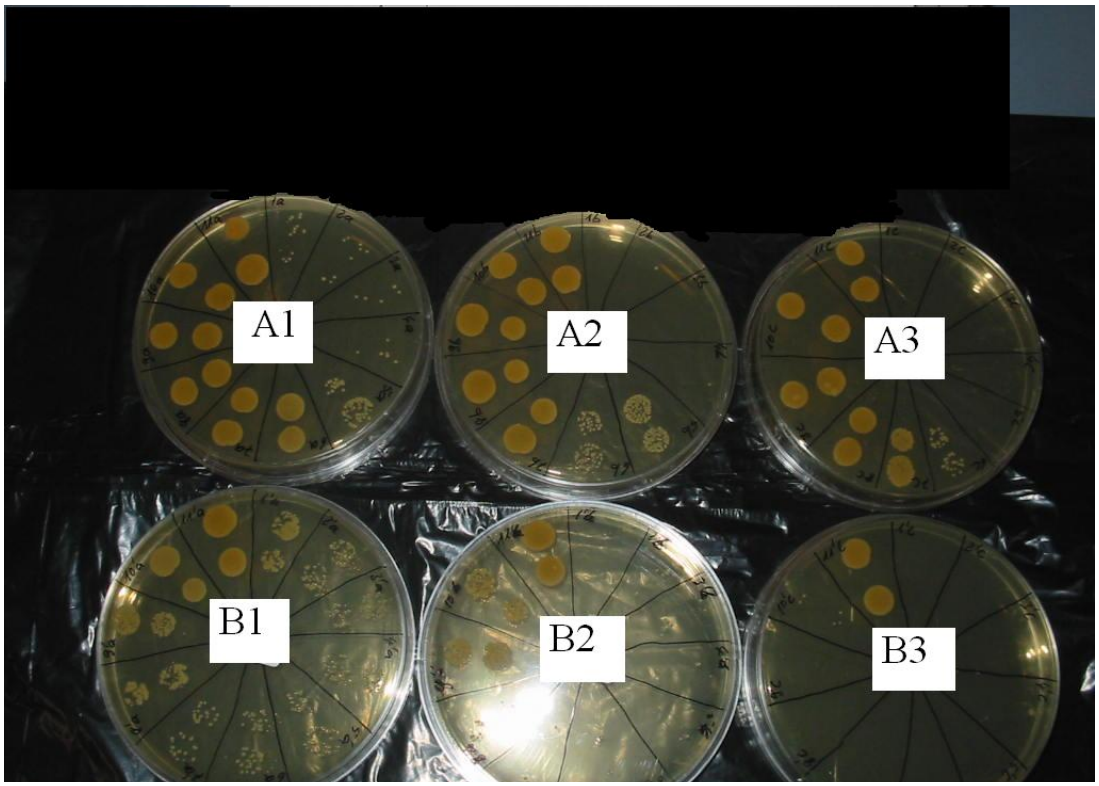
incubation as function of temperature, pH and cell load. The significance of the selected variables was evaluated firstly by the relation between each variable alone and the probability of no growth examined by a likelihood ratio test; the reduction in deviance (-2 times log likelihood) when entering the variable into a model with no other variables was tested against a χ^2 value (Hosmer and Lemeshow, 1989). In addition, the significance of each variable was tested by removing it from a complete model with all variables included. Interactive and quadratic effects of significant main variables were tested in the same way. The final validation of the model was also performed assessing the percentage of correct predictions compared to the observed experimental results.

IV-4 Results

IV-4-1 Minimum inhibitory concentration (MIC)

The use of plate reading (figure IV-3) for the assessment of the activity of the various fatty acids tested, indicated that the absence of turbidity does not necessary means absence of cells. In the example of figure 2, where the two spots in any triangle represent two discharging of the 10μL culture solution, the plates A3 and B3 can easily be taken as no growth results. The error is more probable in the case of A2-b4 respect to the other conditions in the same plate. This result, obtained in broth pH5 and incubation temperature 37°C for 2 hours, show how decanoic acid (B1-B3) totally inactivated *S.aureus* in 9 concentrations of the 10 tested compared to the control in position 11. This method even if time consuming, improve the assessment of case of partial inactivation. The MIC obtained with octanoic, decanoic, lauric linoleic and linolenic acids are presented in table3, a, b, c, d and e respectively.

Figure IV-3: Example of plate spot used to determine inactivation in broth solution of fatty acids Towards *S. aureus*.



The conditions of the example are: 24 hours of incubation at 20°C of the broth solution. The number 1, 2 and 3 are for cell loads of 8, 6 and 4 Log CFU/ ml respectively. A and B are respectively for pH7 and pH6 acids. The subdivisions inside the plates are decreasing concentrations of lauric acid 550 to 0ppm from 1 to 11 (11 being the control)

At pH 6, the antibacterial activity was lower than at pH 5 and in both cases it decreased with the temperature. At 4°C only octanoic acid activity was observed at pH 5 after 24hours especially when the initial cell load was 4 log CFU/g. The influence of cell load and incubation time was observed only at pH 5 where generally the octanoic acid antimicrobial activity increased with incubation time and decreasing inoculum concentration.

Decanoic acid was in general more active than octanoic acid in all the condition tested. At 4°C, total bacterial inactivation was observed only for 4 log cell concentration with the MIC being in these cases 1500ppm, 1500ppm and 200ppm at pH 7, 6, and 5 respectively. In the case of pH6, even though the inactivation concentration was 1500ppm, a cell load reduction higher than 4Log CFU/ml was observed for lower concentrations up to 500ppm (table 3.b). The influence of

incubation time and cell load was less evident at 37°C. The highest activity of decanoic acid was observed at pH 5.

Lauric acid demonstrated higher antimicrobial potential than the two fatty acids previously discussed but with similar dependence of pH and temperature. However, at 4°C cell reduction or total inactivation activity was observed only after 24 hours of incubation. The influence of initial cell load and time of incubation was more evident in this case and the lowest MIC observed was 5ppm after 24 hours of incubation at 37°C, pH5 with 4 log CFU/ml inoculum (table 3.c).

Regarding α-linolenic acid (table 3e), the MICs observed were in general the lowest of all the fatty acids tested. At 37°C, α-linolenic was more active at pH7 than at pH5, especially when tested at 37°C. Lauric acid was more active especially at pH5 and 37°C than α-linolenic was. For α-linolenic the tendency was for inactivation potential to decrease with the pH as the temperature increased, showing a different trend respect to other tested fatty acids. At 4°C only the lowest inoculum conditions were inactivated.

Linoleic acid had very few cases in which it was active on *S. aureus* and generally it was when cell load was 4 Log CFU/ml and pH 7 for high temperatures (20 and 37°C), and for all the tested pH at 4°C (table 3.d). Stearic and oleic acid did not have any inhibitory activity at the conditions tested. In general, the results obtained were similar to that of Kabara et., al. (1972) who found a MIC of 1.79 μ mole/ml (498 ppm) for α-linolenic on *S. aureus*, testing the FFA on an inoculum of around 10^{10} CFU/ml at optimal temperature after incubation for 18 hours. In our experiment, for 10^{8} CFU/ml initial cell load at the same conditions after 24 hours incubation, we found 300ppm. On the other hand, Lee et., al. (2002) observed a surprising low MIC of 50ppm after 72 hours for an inoculum of 10^{6}-10^{7} CFU/ml at 37°C at physiological pH. Zheng, et al., (2005) observed MICs for 10^{4} CFU/ml initial load of *S. aureus* at 37°C pH7.4 -7.5 of 2 and 4 mM for linolenic and linoleic acids respectively, after 18 hours, while at pH7, in our experiment, linoleic acid resulted to be slightly more active than linolenic acid for the same initial cell load only in some conditions, but not in a two fold ratio difference. As in our experiment, Kabara, et al., (1972) didn't find any inhibitory activity for linoleic acid on *S. aureus* for concentrations up to 6 μ mole/ml for cell load of around 10^{10} CFU/ml at 37°C after incubation for 18 hours.

Table IV-3: (a, b, c and d)- Minimum inhibition concentration of octanoic, decanoic, lauric and linolenic acid respectively on *S. aureus* sub-cultured and grown in different environmental condition

a-Octanoic acid

incubation		37°C			20°C			4°C		
incubation		pH 7	pH 6	pH5	pH 7	pH 6	pH5	pH 7	pH 6	pH5
C8:0 2 h	10^8	-	1500	1000	-	-	1500](-)	-	-	-
	10^6	-	1500	800	-	[1500](-)	1500](-)	-	-	-
	10^4	[400](-)	1500	600	-	[1500](-)	1500	-	-	-
C8:0 24 h	10^8	-	1500	1000	-	1500	800	-	-	[1500](-)
	10^6	-	1500	500	-	1500 [600](-)	800	-	-	1500 [800](-)
	10^4	-	1500	500	-	1500 [300](-)	600	-	-	600

-("-" no inhibition up to 1500 ppm)

- "[ ](-)" cell load reduction > 10^4 cfu up to the indicated concentration

b- Decanoic acid

incubation		37°C			20°C			4°C		
incubation		pH 7	pH 6	pH5	pH 7	pH 6	pH5	pH 7	pH 6	pH5
C10:0 2 h	10^8	-	300	100	[1500](-)	1000	-	-	-	-
	10^6	[600](-)	300	100	[1500](-)	600 [300](-)	[200](-)	-	-	-
	10^4	1500	300	100	[1500](-)	300	200 [100](-)	-	-	-
C10:0 24 h	10^8	1500	300	100	1500	300	200	-	-	-
	10^6	600	300	100	1500 [600](-)	300	100	-	-	-
	10^4	600	300	100	1500 [600](-)	300	100	1500	1500 [500](-)	200

-("-" no inhibition up to 1500 ppm)

- "[ ](-)" cell load reduction > 10^4 cfu up to the indicated concentration

c- Lauric acid

Incubation		37°C			20°C			4°C		
Incubation		pH 7	pH 6	pH5	pH 7	pH 6	pH5	pH 7	pH 6	pH5
C12:0 2 ore	10^8	-	[400](-)	20	[500](-)	-	[100](-)	-	-	-
	10^6	800	[400](-)	20 [10](-)	[600](-)	-	[50](-)	-	-	-
	10^4	600	400	10 [5](-)	500	[50](-)	100 [40](-)	-	-	-
C12:0 24 ore	10^8	-	800	10	[500](-)	[100](-)	[100](-)	-	[400](-)	[600](-)
	10^6	600	400	10	600	200	50	[1000](-)	1000	[600](-)
	10^4	600	300	5	500	100	20 [10](-)	[300](-)	800	300

-("-" no inhibition up to 1500 ppm)

- "[ ](-)" cell load reduction > 10^4 cfu up to the indicated concentration

d- Linoleic acid

Incubation		37°C			20°C			4°C		
Incubation		pH 7	pH 6	pH5	pH 7	pH 6	pH5	pH 7	pH 6	pH5
C18:2 2 ore	10^8		-	-	-	-	-	-	-	-
	10^6	[50](-)	-	-	-	-	-	-	-	-
	10^4	50 [10](-)	-	-	-	-	-	-	-	-
C18:2 24 ore	10^8	-	-	-	-	-	-	-	-	-
	10^6	50	-	-	[15](-)	-	-	-	-	-
	10^4	15	-	-	30	-	-	50	50	600

-("-" no inhibition up to 1500 ppm)

- "[ ](-)" cell load reduction > 10^4 cfu up to the indicated concentration

e- linolenic acid

Incubation		37°C			20°C			4°C		
Incubation		pH 7	pH 6	pH5	pH 7	pH 6	pH5	pH 7	pH 6	pH5
C18:3 2 ore	10^8	300 [25](-)	[25](-)	-	-	-	-	-	-	-
	10^6	25	[25](-)	-	[25](-)	[25](-)	-	-	-	-
	10^4	25 [20](-)	25 [10](-)	[15](-)	50 [25](-)	[25](-)		50	50	-
C18:3 24 ore	10^8	300[20](-)	25	1000	50	[15](-)	[50](-)	-	-	-
	10^6	25 [20](-)	25	1000	50 [25](-)	15 [10](-)	25 [10](-)	-	-	-
	10^4	20	25 [10](-)	25	25 [20](-)	10	10 [5](-)	50	50	15 [10](-)

-("-" no inhibition up to 1500 ppm)

- "[ ](-)" cell load reduction > 10^4 cfu up to the indicated concentration

IV-4-2 No growth probability

Staphylococcus aureus was inoculated at different concentrations in broth characterized by different combinations of pH and Temperature, containing free fatty acids (FFAs) in quantities varying from 0 to 1500 ppm. The growth or no growth of the cells was assessed by plating and recorded as "0" and "1" respectively. In table IV-3a and IV-3b, the most suitable logit equations obtained applying the logistic regression are represented for 2 hours and 24 hours incubation respectively.

Table IV-3a: Parameters coefficients and standard errors (se) of the logit model describing the influence of temperature (T), inoculum (Load) and pH on the growth and no growth probability of *S. aureus* in the presence of selected fatty acids after 2 hours of incubation

Parameters	**C8:0 2h**	**se C8:0**	**C10:0 2h**	**se C10:0**	**C12:0 2h**	**se C12:0**	**C18:3 2h**	**se C18:3**
intercept	29.3212	8.6339	-23.092	4.13537	-160.556	5.53411	-15.733	1.56621
T	1.1967	0.2893	1.38772	0.17018	5.35542	0.04751	0.07418	0.01094
Load	-1.3352	0.4042	-0.5618	0.09028	-1.11773	0.14983	-1.2552	0.11466
pH	-16.414	4.0576	3.44029	0.65314	23.2044	0.6834	2.90803	0.25502
[FA]	0.02281	0.00570	0.00489	0.00260	-0.02240	0.00551	0.00199	0.00032
T*pH			-0.2118	0.02738				
T*[FA]			0.00013	0.00003	0.00021	0.00006		
pH*[FA]			-0.0009	0.00041	0.00325	0.00072		
χ^2 pearson	41.36		486.730		304.80		424.8	
Max. likelihood	-18.22		-195.87		-117.60		-198.25	
% good classification	96.25		85.4		88.15		82.36	

Table IV-3b: Parameters coefficients and standard errors (se) of the logit model describing the influence of temperature (T), inoculum (Load) and pH on the growth and no growth probability of *S. aureus* in the presence of selected fatty acids after 24 hours of incubation

Parameters	**C8:0 24h**	**se C8:0**	**C10:0 24h**	**se C10:0**	**C12:0 24h**	**se C12:0**	**C18:3 24h**	**se C18:3**
intercept	550.775	143.749	10.89332	2.04321	11.96227	2.85260	0.13731	0.82151
T	1.17813	0.32482	0.59568	0.10522	0.59688	0.09620	0.03001	0.01226
Load	-1.38861	0.23202	-0.62553	0.08932	-1.53895	0.13609	-1.1217	0.08260
pH	-109.515	28.67053	-1.85380	0.35367	-1.44529	0.50102	0.80471	0.12996
[FA]	-0.34514	0.09239			-0.00519	0.00260	0.00014	0.00042
T*pH	-0.27899	0.07234	-0.09121	0.01802	-0.08553	0.01673		
T*[FA]	0.00052	0.00010	0.00024	0.00003	0.00017	0.00003	0.00015	0.00002
pH*[FA]	0.06950	0.01847			0.00124	0.00046		
χ^2 pearson	267.01		701.44		554.25		730.74	
Max. likelihood	-99.79		-200.44		-199.28		-274.24	
% good classification	89.45		91.35		88.81		86.91	

All the models obtained included all the studied parameters and the percentage of good classification were higher than 85 % unless in the case of C18:3 after 2 hours of incubation. The highest difficulty was that of modelling the bounder between the growth and no growth region, especially when this limit was near 0ppm of fatty acid in the medium. Nonetheless, the models give a good picture of the trends and probabilities of inactivation when in The presence of fatty acids as antimicrobial.

A very good precision of the no growth probability (NGp) was obtained in the presence of octanoic acid. The NGp of 4 Log UFC/ml of *S. aureus* after 2 hours incubation at pH5 was null at temperature lower than 15°C (figure IV-4). Moreover, the NGp depended also on the pH (figure IV-5). In fact, as the pH decreased, the minimum FFA concentration required to a NGp > 0 also decreased. Lets call no growth transition value (NGTV), the fatty acid concentration needed to pass from a situation of NGp>0 to NGp=1, it can be observed that in the case of octanoic acid, this interval was very narrow. In other words, only about 250 ppm was needed to obtain the total inactivation, after the minimum required for partial inactivation. The increase of the cell load had the effect of lowering the No growth probability and increase the FFA concentration needed to obtain the a NGp>0 (figure IV-6).

Figure IV-4 No growth probability after 2 hours of incubation of 4Log UFC/ml of *S. aureus* at pH 5 as function of temperature in the presence of octanoic acid

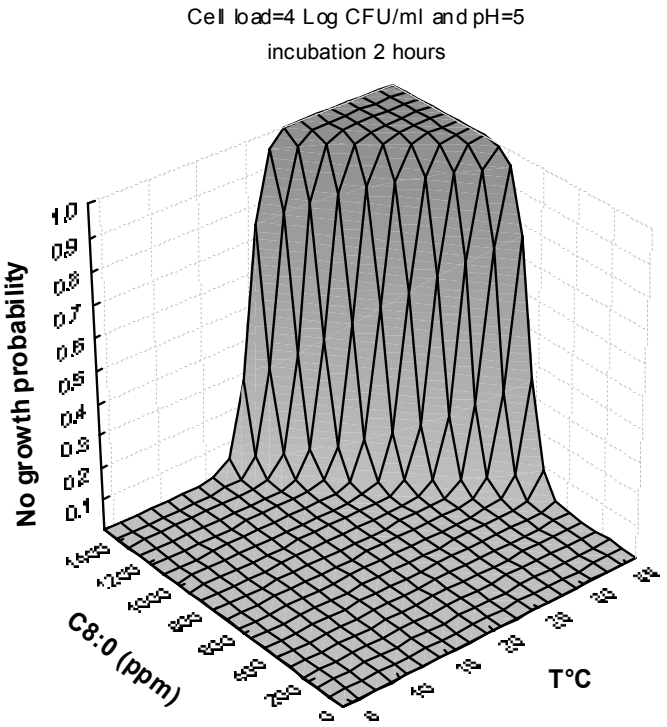


Figure IV-5 No growth probability after 2 hours of incubation of 4Log UFC/ml of *S. aureus* at 36°C 5 as function of pH in the presence of octanoic acid

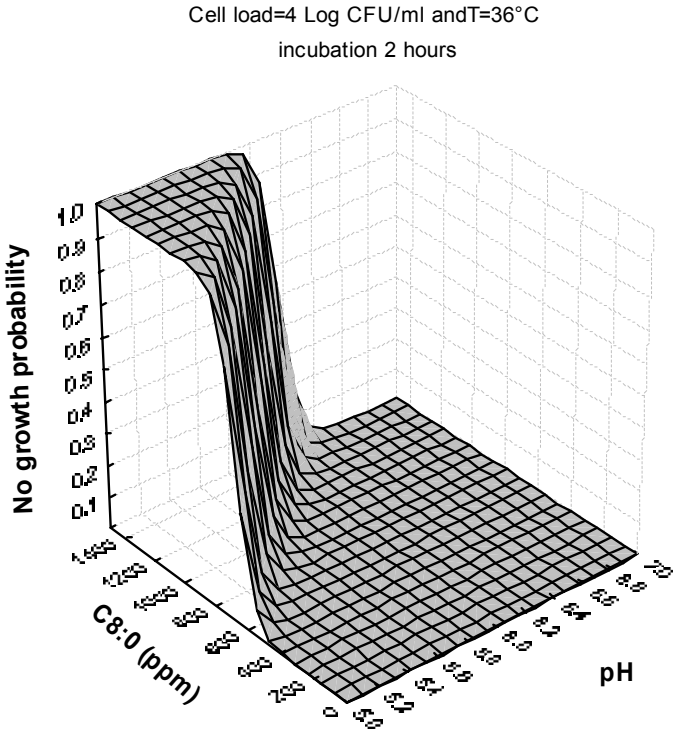
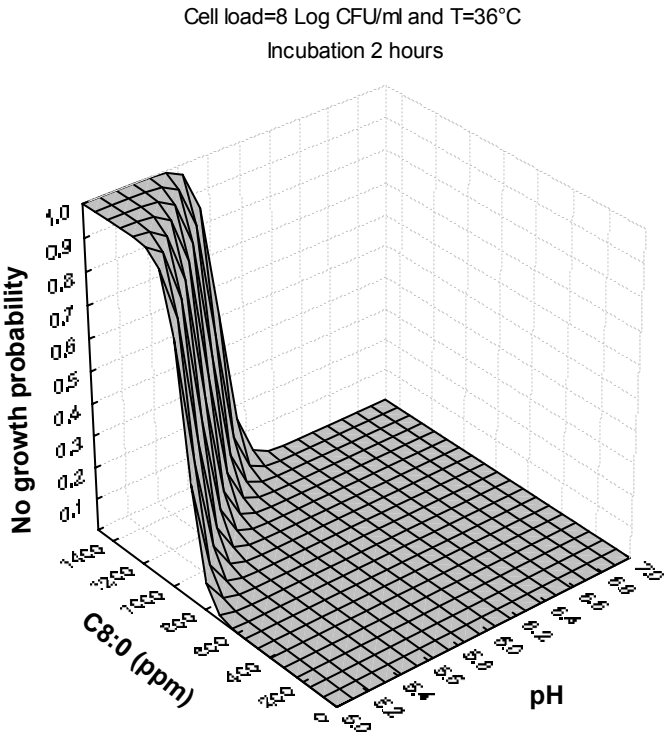


Figure IV-6 No growth probability after 2 hours of incubation of 8 Log UFC/ml of *S. aureus* at 36°C as function of pH in the presence of octanoic acid



Regarding the effect of C10:0 on *S. aureus*, the NGp observed was in general higher than that of C8:0 in the same conditions. At pH 6, only a concentration of C10:0 higher than 1400 ppm and an incubation temperature higher than 36°C allowed a NGp of 1 in the presence of 8Log CFU/ml of *S. aureus* (figure IV-7). In this same conditions, no probability of inactivation was observed under 15°C. The decrease of pH together with the increase of the decanoic acid concentration had the effect of enhancing the NGp (figure IV-8). On the contrary to what observed with Octanoic acid, the NGTV was very high for decanoic acid, even though the minimum concentration required to have a NGp>0 was always lower than that of C8:0 in the same conditions. The reduction of the cell load increased the NGp and the no inactivation temperature zone.

Figure IV-7: No growth probability after 2 hours of incubation of 8 Log UFC/ml of *S. aureus* at pH 6, as function of temperature in the presence of decanoic acid

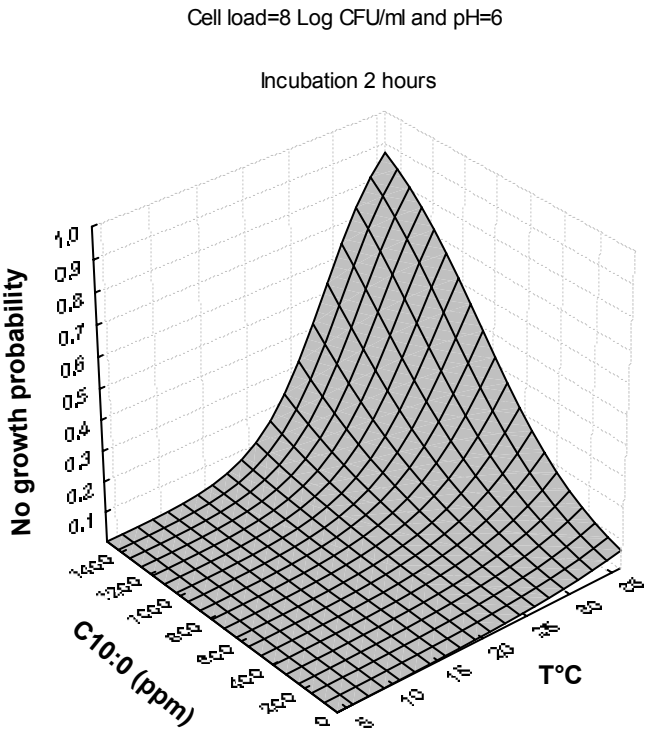
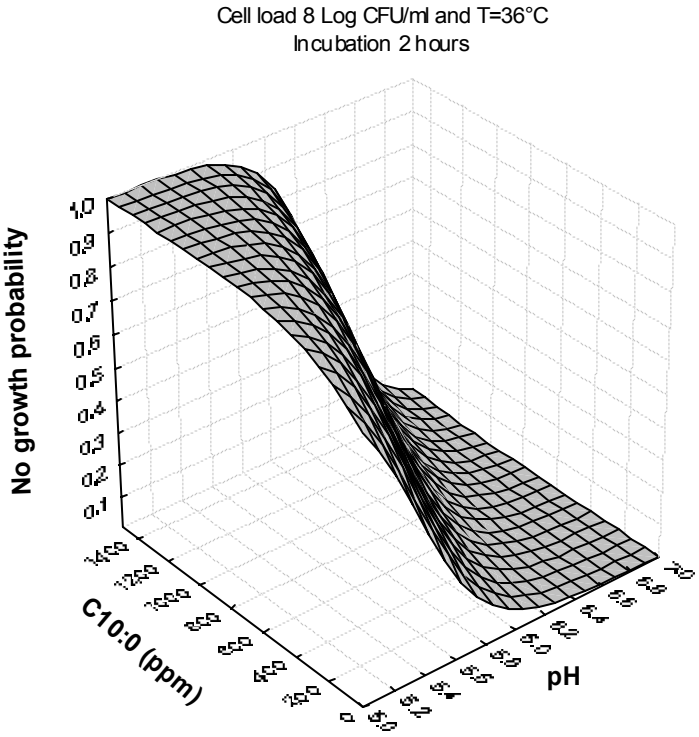


Figure IV-8: No growth probability after 2 hours of incubation of 8 Log UFC/ml of *S. aureus* at 36°C, as function of pH in the presence of decanoic acid



In figure IV-9 and figure IV-10 the NGp after 2 hours of incubation of 4Log UFC/ml of *S. aureus* at pH 5 and pH 7, respectively, are reported as function of temperature. At pH 5, the inactivation began only when the incubation temperature was higher than 30°C (figure IV-9). The comparison of figure IV-9 and figure IV-4 indicates that lauric acid did not cause any inhibition before 30°C, whereas in the presence of octanoic acid at 20°C there was a principal of inactivation at 1400ppm. Moreover, the no growth transition value was very low in the presence of lauric acid with respect to octanoic acid. Increasing the pH while maintaining the other conditions as in figure IV-9, the NGp was zero under 400ppm of lauric acid (figure IV-10). The increasing of the cell load to 8Log CFU/ml at pH 7 had the effect of increasing to 1000ppm the concentration under which the NGp is null (figure IV-11). In none of the previous conditions the NGp was equal to 1 after 2 hours of incubation, while after 24 hours, only few conditions could guarantee the total inactivation of *S. aureus* after an inoculum of 8 log CFU/ml (figure IV-12).

Figure IV-9: No growth probability after 2 hours of incubation of 4 Log UFC/ml of *S. aureus* at pH 5, as function of Temperature in the presence of lauric acid

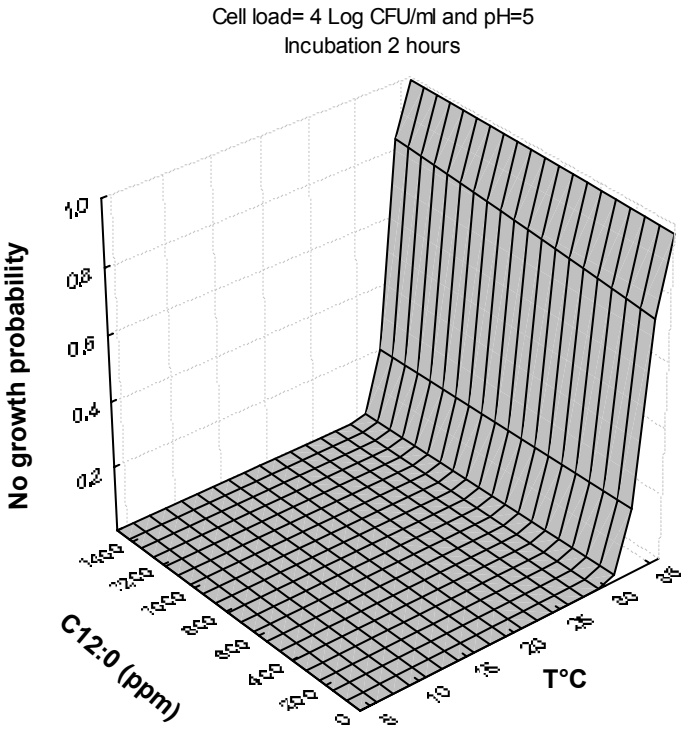


Figure IV-10: No growth probability after 2 hours of incubation of 4 Log UFC/ml of *S. aureus* at pH 7, as function of Temperature in the presence of lauric acid

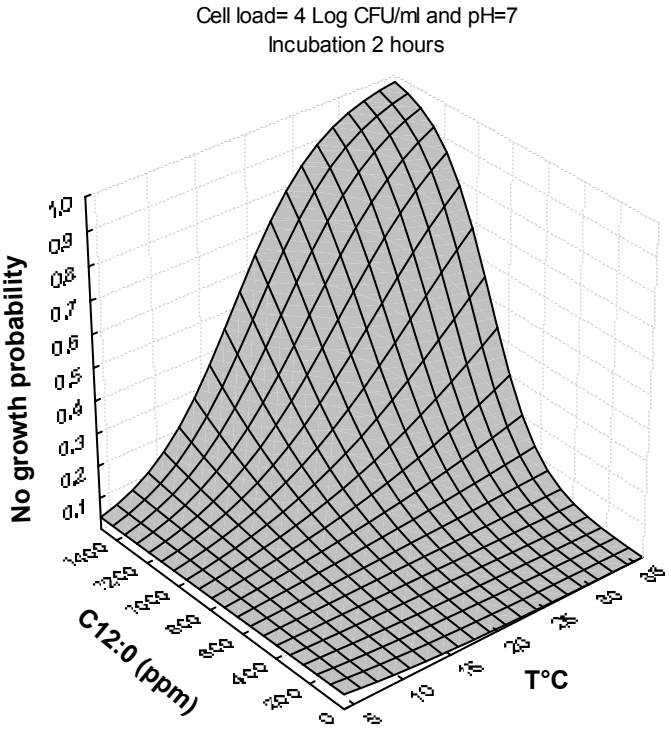


Figure IV-11: No growth probability after 2 hours of incubation of 8 Log UFC/ml of *S. aureus* at pH 7, as function of Temperature in the presence of lauric acid

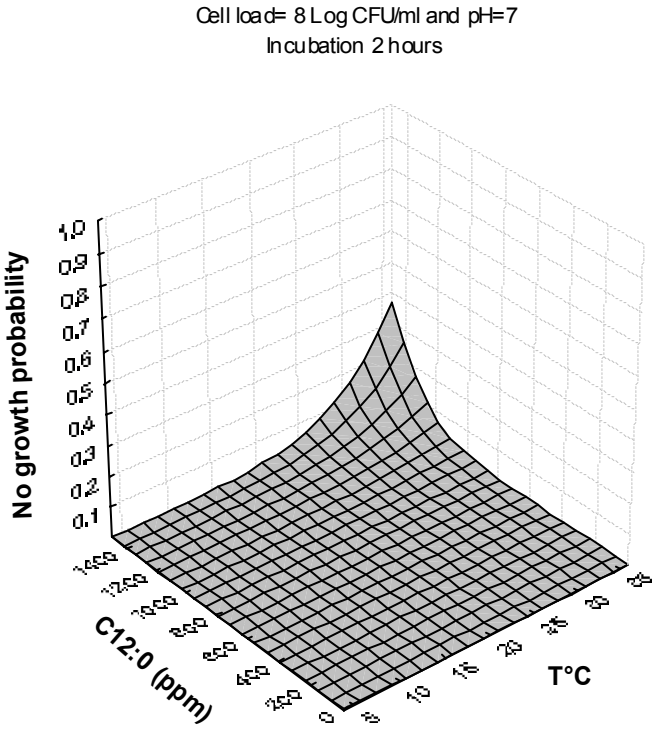
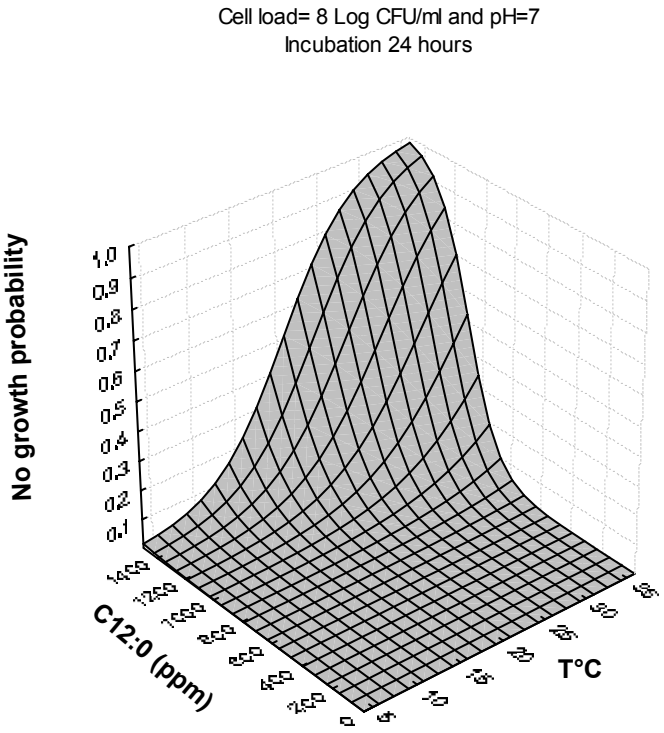


Figure IV-12: No growth probability, after 24 hours of incubation, of 8 Log UFC/ml of *S. aureus* at pH 7, as function of Temperature in the presence of lauric acid



C18:3 had a trend opposite to that of the other FFAs for what regarded the synergistic effect of pH. In fact, at pH 5, even with a cell load of 4 Log CFU/ml, the highest NGp obtained was 0.13 after 2 hours. The NGp of 8 Log CFU/ml of *S. aureus* in the presence of C18:3 at 36°C, was higher at pH 7 than at pH 5 (figure IV-13). Any partial inhibition after 2 hours of incubation at 36°C began when the pH was higher than 5.6 and alpha-linolenic concentration higher than 1400 ppm. After 24 hours, there was a considerable increase in the probability of inactivation at 36°C (figure IV-14). Still after 24 hours, at 36°C and pH 7, the NGp was equal to 1 only when the temperature was higher than 25°C and the concentration of alpha linolenic acid higher than 1000 ppm (figure IV-15).

Figure IV-13: No growth probability, after 2 hours of incubation, of 8 Log UFC/ml of *S. aureus* at 36°C, as function of pH in the presence of alpha linolenic acid

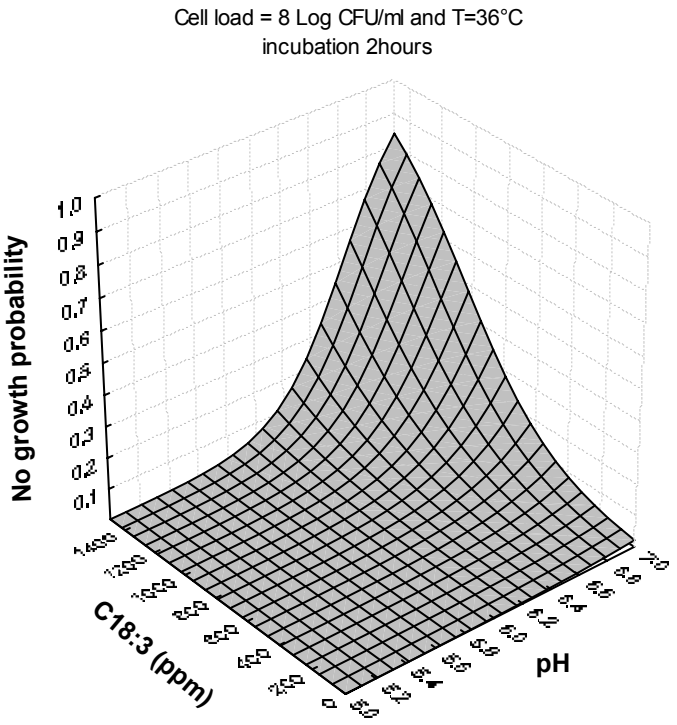


Figure IV-14: No growth probability after 24 hours of incubation, of 8 Log UFC/ml of *S. aureus* at 36°C, as function of pH in the presence of alpha linolenic acid

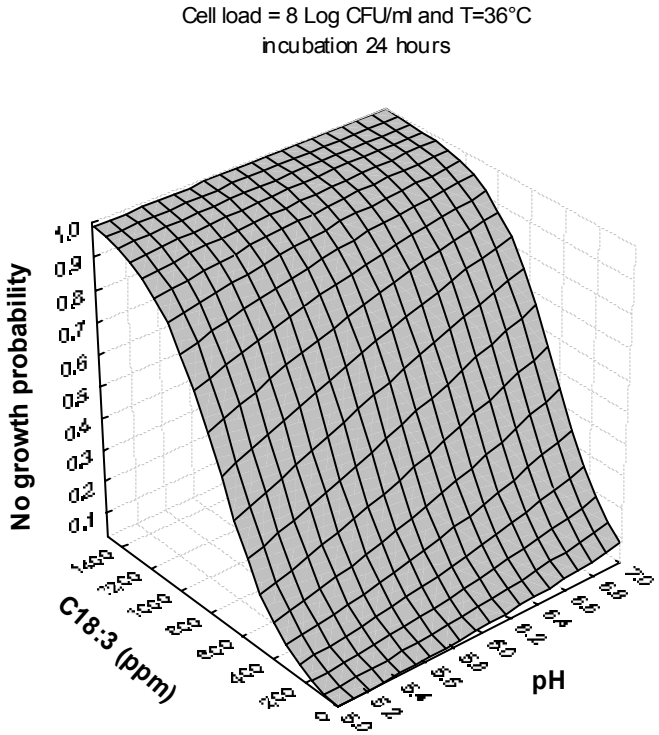
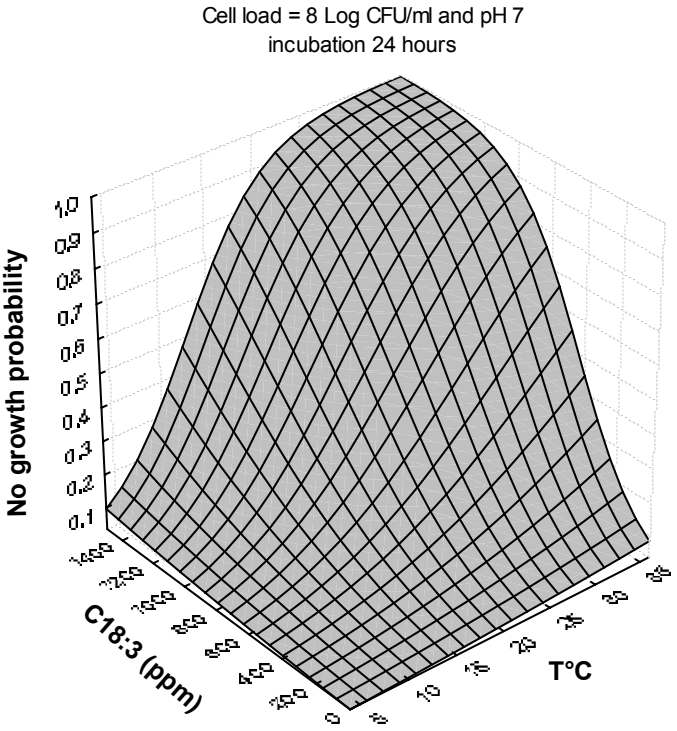


Figure IV-15: No growth probability after 2 hours of incubation, of 8 Log UFC/ml of *S. aureus* at pH 7, as function of temperature, in the presence of alpha linolenic acid



IV-5. Discussion

In this study, the MIC of selected free fatty acids on *S. aureus* and their No growth probability in the presence of the same FFAs in different environmental conditions, initial cell load and time of incubation were assessed. An experimental protocol was adopted in order to avoid false positive results and the influence of cell adaptation to the new environment temperature on the fatty acid activity. For this later reason, the cells were incubated for 24 hours in the conditions in which the fatty acid had to be tested. Mechanical dissolution of the FFA using ultraturax and ultrasound did not show any difference compared to the use of solvent in the cell inactivation and the survival cell growth dynamics. This indicates that the dispersions obtained mechanically, can for the period in which they are stable, have the same result as solvents such as ethanol and DMSO diffusion of FFA. In fact, composition, temperature and energy used in mechanical dispersion can influence the

condensation process of the micelles of oil in water (Abisma et al., 1999; Chanamai et al., 2002; Roland et al., 2003). The difficulty of maintaining sterility is one of the big disadvantages in using mechanical dispersion of FFA. Regarding the two solvents, ethanol and dimethylsulfoxide (DMSO) tested at 1% (v/v) of the growth medium, cells grown in DMSO showed a different growth dynamic respect to those of the control and supplemented with ethanol. In particular, the cell load after 26 hours of incubation was lower in DMSO. Anyway, the concentration used, that correspond roughly to a molar fraction of 0.0026, does not have any influence on the cell wall as indicated by Chen et al., (2004), who observed a remarkably promotion of lamellar crystal phase by DMSO on lipid bilayer only at molar fraction higher than 0.37. The cell load reduction observed in the presence of DMSO could then be attributed to the high polarity of DMSO, which can chelate some substances that become limiting as the bacterial population increase. In the case of Brij 58, which is a nonionic surfactant of the polyethylene glycol family, the *S. aureus* growth dynamic was similar to that recorded in the presence of DMSO, indicating a similar effect. The solubility of FFA depends on the type and quantity of solvent in solution (Maeda et al 1996), while pKa especially in the case of long-chain fatty acids, depends on the degree, type and position of the unsaturation (James et al., 2002).

The temperature influence on the MICs was evident in all the fatty acids that showed inhibition activity. The temperature can influence both the cytoplasmic membrane Characteristics and the fatty acids physical properties (Denich et al., 2003; James et al., 2002). The cells, to adapt the membrane fluidity to the changing environment temperature, can modify the fatty acid and the phospholipids composition (Russel 1984; Diefenbach et al., 1992; Heipieper et al. 1992). In the case of *S. aureus*, Bhakoo and Herbert, (1980) did not observed any significant change in the fatty acid composition when grown at 25 and 37°C while it was the case for psychrotrophilic *Vibrio* sp. This suggest that *S. aureus* can regulate the fluidity of its membrane in this range of temperature without necessarily changing the composition of the membrane fatty acids as we expect to happen when passing from 4°C to 20-37°C. In our study, C8 and C10 did not have much differences in their MIC values at 20 and 37°C, while this was the case for C12 and C18:3. Low temperatures cause the transition towards the gel phase state, while moving to high temperature enhance the fluidity due to increased rotational motion of the glicerophospholipids, especially about the C-C bonds of the acyl chain.(Hazel and Williams, 1990). On the other hand, Høyrup et al. (2001) found in model system that the partitioning of capric, lauric, myristic and palmitic acids did not depend on the lipid membrane phase behaviour (low-temperature gel and high temperature fluid-membrane phase).

However data concerning partitioning of FA within cell membrane in relation to the temperature are lacking. Moreover it is necessary to take into consideration the fact that the diffusion of fatty acids in the medium is strongly affected by their physical state and the gelification phenomenon of cell wall at low temperatures. So the antimicrobial activity of these saturated fatty acid can be related to their capacity of braking the bacterial internal ionic equilibrium when they can easily pass through the cell wall (fatty acids with less than 12 atoms of carbons), or cause disorder in the membrane bilayer molecular arrangement, increasing the permeability. The more the fatty acid carbon chain is long, the higher the hydrophobicity, and their accumulation into the lipid bilayer alters the hydrogen bonding and the dipole-dipole interaction between acyl chains, disrupting the organization of the glycerophospholipids within the membrane (Antunes-Madeira et al., 1995). Antimicrobial capacity is also correlated to the partitioning capacity of the compound, and in general when the Log_{10} $P_{O/W}$ (1-octanol-water partition) is below 5, the compound is assumed to have a good affinity to bacterial membranes (Heipieper et al. 1994; Antunes-Madeira et al., 1995) and a theoretically antimicrobial capacity. While for short and medium chain fatty acids, their acidifying effect in the cytoplasm and the disrupting action on the cell membrane seems more appropriate as antimicrobial effect, for unsaturated fatty acids, the damage on the bacterial membrane and the effect on the fatty acids synthesis are more plausible. In fact, Zheng et al. (2005) demonstrated that unsaturated FA (palmitoleic, oleic, linoleic, linolenic and arrachidonic acids) as their antimicrobial mechanism, inhibited the bacterial enoyl-acyl carrier protein reductase (FabI), a key enzyme in the bacterial fatty acids biosynthesis.

Between the fatty acids we tested, linoleic and linolenic acid showed a different behavior to pH respect to caprylic, capric and lauric acids. The mechanism of adaptation to acid environment for bacterial, include also the increase of the proportion of long chain mono-unsaturated fatty acids in the membrane, with a concomitant decrease in saturated fatty acids (Fozo et al. 2004 and Fozo et al. 2004). Increasing temperature and decreasing pH could consequently have a synergistic effect in enhancing the cell membrane fluidity and hence the possibility of disrupting effects and of the FA partitioning. Our results show that when pH neared the pKas of the short chain FA, they were more active. Linoleic and linolenic acid which pKa were higher than the pH tested, and so the soluble FA concentration was mostly in the undissociated form with more affinity to the membrane in all the conditions tested. The low activity of these two unsaturated FA at pH 5 respect to pH 7 when tested at 37°C, could suggest that *S. aureus* already adapted to the acidified environment as in our case, has a higher tolerance to unsaturated fatty acids that may be used in the metabolic pathway.

The difference observed in the MIC values after 2 and 24 hours of incubation can be attributed to the rate of activation associated to the type of inhibition activity of the different FA. Hamilton (1998) observed that the constant rate of adsorption of FA monomers to phospholipids bilayer vesicles is extremely fast and quantitative. This means that the rate of inactivation is not correlated to the last aspect, but to consequences of their presence in the membrane that can lead the bacteria to dead at different rates. In the case of short chain fatty acids, it could be the rate of penetration of non-bacterial friendly compounds that has to penetrate the cell as ions. This rate will then depend on their concentration (es. protons will be more present at low pH and take advantage of the positive gradient to enter the cell with a higher rate). Regarding long chain unsaturated fatty acids, the rate could be related to the disturbance and interference of the bacterial fatty acid synthesis.

The influence of cell concentration is of difficult interpretation as in some cases the MIC was not proportional to the inoculum size. Even if in general, FFAs were more effective as antimicrobial at low initial cell load, the hypothesis of the population MIC being the some of the individual cell MICs can not be made on the bases of the results of our experiment.

The discussion made on the results of the MIC, is based on the assumption that the system was perfectly controlled. But the differences in the results observed in literature as previously mentioned, indicate that uncertainty in any biological experiment is the sum of natural and operators errors. When a system can not be totally controlled or is not totally understood, then the measured effects of an external variable on it can not be considered as an absolute value. Cytoplasmic membranes are the selective barriers that protect bacterial from the environmental changes. Any changes in the environment is likely to cause changes in the bacterial membrane composition, physical properties and/or metabolic pathway (Russel 1984; Diefenbach et al., 1992; Heipieper et al. 1992; Langner and Hui, 2000; Denich et al., 2003; James et al., 2002 ; Ndagijimana et al., 2003). Dealing with pathogens, the main goal is their total destruction and not the growth reduction or delay. This objective gives only two possible outcomes; “Growth” or “No growth” when antimicrobials are tested against pathogens. Logistic regression in this context is a suitable way of modelling this type of dichotomous data as function of the system variables. The probability of total inactivation (no growth probability) of *S. aureus* with different FFAs (C8:0; C10:0; C12:0 and C18:3) at different temperatures and pH was assessed after 2 and 24 hours of incubation. At refrigeration temperature (in general under 10°C), the NGp was zero or near to zero. Medium chain free fatty acids like C8:0 demonstrated to have a threshold value over which very low increase with

respect to the other FFAs tested was needed to obtain a NGp of 1. This may be a consequence of the higher solubility and partitioning (see $LogP_{O/W}$ in table IV-1) of C8:0 with respect to the other FFAs tested. When the FFAs diffuse into the bacterial membrane, the internal pH is lowered and the membrane permeability is disturbed until the effect reaches a critical value, while the cells try to compensate this by modifying the membrane composition or their metabolism. It can be assumed that this critical value is probably related to individual cells within the population and determine a resistance distribution. Hence the skewness, kurtosis and the mean of the critical value distributions within a population will determine the magnitude of the no growth transition value (NGTV); in other words the concentration needed to pass from partial to total inhibition. The medium chain fatty acids tested showed a different inhibition trend in relation to the pH with respect to C18:3. Taking into consideration the physical properties of these FFAs, it can be said that all the pH tested were below the pKa value of C18:3, indicating that the major part of the compound was in the most active undissociated form unlike medium chain FFAs that have a pKa around 4.9. It is also known that the dissociated form is more soluble but have less affinity with the lipid bilayer. So reducing the pH to 5 may have contribute to reduce the solubility of C18:3 and its availability. Moreover if the mode of action of C18:3 and other polyunsaturated fatty acids is mainly due to the inhibition of the fatty acid biosynthesis, an in particular of the FabI enzyme (Zheng et al. 2005), it can be suggested that the low pH reduce the rate of reaction between the inhibitor and the protein, causing a reduction of the inactivation capacity.

IV-6 Conclusion

The minimal inhibitory concentration of FFAs is not a stable value and does change with the environmental conditions surrounding the bacteria. The mechanism of inactivation seems not to be the same for all free fatty acids and depending on their physical properties. For a good overview of antimicrobial compounds activity it is important to test in different environmental conditions. The application of logistic analysis has demonstrated to be a useful tool in the study of antimicrobial compounds, in particular when only growth and no growth are the expected results. The combined MIC study and probabilistic approach, can ensure a better understanding of the efficacy and suitability of use of the FFAs in different conditions.

IV-7 References chapter IV

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CHAPTER V

EFFECTS OF NON-INHIBITORY CONCENTRATIONS OF SELECTED FATTY ACIDS ON THE GROWTH PARAMETERS OF *Staphylococcus aureus* POPULATION AND ON THE DISTRIBUTION OF THE LAG TIMES OF ITS SINGLE CELLS

V-1 Introduction

Staphylococcus aureus is a crucial factor in health care, both from medical and food safety points of view. It is a versatile bacterium which is very successful in establishing itself as a commensal in various niches, in human and animals (Lowy, 1998). It has been estimated that approximately 185,000 cases of staphylococcal food poisonings occur each year in the USA, the vast majority of which goes unreported (Mead et al., 1999). In meat products, the occurrence of pathogens is related to the hygienic conditions of animal farming, slaughtering, deboning and conditioning. The main ways of eliminating the presence or preventing the growth of pathogen microrganism in food are: i-heat treatment, ii-lowering the pH by natural fermentation and/or by the use of low acid salt compounds like acetate and benzoate, iii-using antimicrobials generally considered as safe, iv-using protective atmosphere packaging. The critical point of all these techniques is the fact that they can always be used only on products that are undergoing a certain transformation. Fresh products (es. Fresh meat) are more problematic for the fight against food pathogens. The best case, one may dream of, is a product that naturally contains antimicrobial compounds and hence self protecting. But a part from the case of some low acid foods, other fresh food products need to be protected by external actions. Among compounds present in meat, the fatty acids (FA) an their esters, mainly mono and diglycerides, can be regarded as potential bactericides and/or bacteriostatics. Some applications of these compounds as preservatives in foods have already been reported (Kabara, 1993; Isaacs et al., 1995; Hinton and Ingram, 2000; Lee et al., 2002; Ababouch et al., 1994; Coroller et al., 2005). The limiting aspect of meat fatty acids is that they are generally present in the form of glycerol esters, and only very low quantities are free or in the form of mono and diglycerides. The minimal inhibitory concentration (MIC) of some fatty acids against a wide range of bacteria have been reported (Kabara et al., 1972 and Lee et al., 2002), and, if they are

compared to the relative amount of these compounds found in meat and meat based products, in most of the cases their concentration is not sufficient to cause total inactivation. It is then important to evaluate the antibacterial potentialities of these low concentrations on the pathogenic microflora.

The growth of a microrganism in an environment is affected not only by classical factors such as temperature, pH, Aw, but also by the presence of compounds that can interact with them physiologically or metabolically and influence their growth. Studying the effect of such compounds on the growth of microrganism is important for future predictions of microrganism responses. Microbiologists have gained, in their collaboration with mathematicians and statisticians, many tools that can help them to describe and compare the behaviour of microrganisms in different environments. To describe their growth as a function of time, many models have been proposed, with the common characteristic of having a sigmoid shape. The Gompertz and logistic equations, which are considered as first generation of empirical models, fit well to bacterial curves (Zwietering, 1990; McMekin, et al., 1993) and are useful for empirical evaluations. A limit of these models is that they just describe a set of data without taking into account the mechanism that generates them (Baranyi and Pin 2004). During the last decades, a new generation of models with increasing mechanistic approach have been proposed for the modelling of bacterial growth curves. Among them, the Baranyi model (Baranyi et al., 1993; Baranyi and Roberts, 1994), Hills model (Hills and Wright, 1994) and Buchanan (Buchanan et al., 1997) model are those most discussed in literature (McDonald and Sun, 1999; Grijspeerdt and Vanrolleghem, 1999; McKellar et al., 2004; Peleg, 2006). Quite all the sigmoid-like models predict the specific growth rate of the bacterial growth with an acceptable accuracy. The difference among them is related to the approach of defining the lag phase. In the model of Hills and Wright (1994), it is defined as the time for the synthesis of chromosomal and non chromosomal materials. In the Baranyi model the lag phase is considered as the time necessary for the cell to accumulate or synthesise an unknown component "q" related to the physiological state of the cell. This 'q' substance is assumed to be necessary to begin the division, (Baranyi and Roberts, 1994). Another improvement of this last model with respect to other mechanistic models is the simultaneous modelling of the specific growth rate and Lag together with the applicability to time-dependent environment.

The common assumption of all the models discussed previously is the consideration of the microbial population responses as homogeneous. This is obviously a very idealistic assumption The only fact that in a population, new births are not simultaneous, is already an element of randomness associated to the individual cell that may be due to the cells or the measurement. The reasons for this

assumption have been believed, for many years, the low sensibility of the techniques used to study the microbial growth and responses, that were not able to target the individual cell. The availability of automatic microbial growth reading instruments like the Bioscreen and flow cytometer have permitted the study of very low concentrations of cells and even single cells (when applying probabilistic approaches), (Stephens et al., 1997; McKellar and Knight, 2000; Métris et al., 2003). Using bacterial data obtained from Bioscreen readings, Baranyi and Pin, (1999) developed a numerical method to estimate the variability of the lag time of individual cells from the detection times. This variability cannot be identified from the traditional lag assessment using data from population viable count curves. (Baranyi, 2002).

The error in the determination of the population growth parameters is likely to be higher when estimating the lag than the specific growth rate, because the variability of the time to the first division (which account for the population lag) is very high with respect to that of the subsequent divisions (accounting for the specific growth rate) (Métris et al., 2005). It is then important to take into account the distribution of the population lag time in order to model with more accuracy the probability of survival and growth at low bacterial concentrations (Baranyi, 2002). Kutalik et al., (2005) analysed the connection between stochastic and deterministic modelling of microbial growth, and concluded that the shape and of the individual lag distribution cannot be retrieved from information about the population growth. This variability in the individual cell lag time is a fundamental aspect in risk assessment, as it introduces a stochastic variable in the prediction of the time by which a food stuff becomes unsafe.

In response to the difficulty of deriving single cell lag times from population data, Elfwing et al., (2004) introduced a powerful technique based on a flow chamber microscope system, that enable the observation of the growth and division of thousands of individual bacteria simultaneously. With the help of this new tool, the problem of measuring the population lag can be looked the other way round; this means by measuring directly the time to first, second, …, and n-th division times of thousands of single cells and using these distribution to simulate the population growth curves (see Pin and Baranyi, (2006) for the theory and a case study of this approach). The time to the first division of the single cells is considered as the sum of the lag and first generation time. For the sake of simplicity, we will refer to it as the single cell lag time.

The second step in studying the response of microrganism is the assessment of the correlation between the environment variables and the growth parameters. Models on the effects of variables on the microrganism growth parameters are called secondary models. Generally secondary

models, that are available in literature, are said to be incomplete, because they do not take into consideration all the variables that are present in food (Ross and Dalgaard, 2004). Most of those models are hence derived from experimental design where only the target parameters of the environment to be studied are taken into consideration. In order to develop a consistent framework that enables us to understand and predict the microbial ecology of foods, it is desirable to integrate the patterns of microbial behaviour revealed in predictive modelling studies with the knowledge of the physiology of microorgnism and physico-chemical processes and phenomena that occur in foods and food processes (Ross et al., 2000). It becomes obvious then to distinguish empirical deterministic models from mechanistic secondary models. In the first case, the model provides only a mathematical relationship between the environmental parameters and the microbial growth parameter studied. In the second case, there is an attempt to introduce in the equations, terms that are related to the microbial behaviour proven theories. The use of deterministic secondary models can only be a first step towards a mechanistic approach, depending on the understanding of the biological explanations of the correlations found.

With this mathematical background, the aims of this part of the work were: i- to assess the influence of non inhibitory concentrations of Caprylic acid (octanoic acid, C8:0), Capric acid (decanoic acid, C10:0), Lauric acid (dodecanoic acid, C12:0) and alpha linolenic acid (c-9,c-12,c-15-octadecatrienoic acid, C18:3 n-3) together with temperature and pH on the specific growth rate of *S. aureus*. ii- to evaluate the distribution of the *S. aureus* single cell times to first division and successive generation times, when in presence of caprilic, capric and alpha-linolenic acids. iii- to assess the effect of different inoculum size on the population growth parameters using the observed single cells time to first division and second generation time distributions.

V-2 Materials and Methods

V-2-1 Influence of fatty acid concentration, temperature and pH on the specific growth rate

V-2-1-a Preparation of fatty acids stock solutions and growth medium

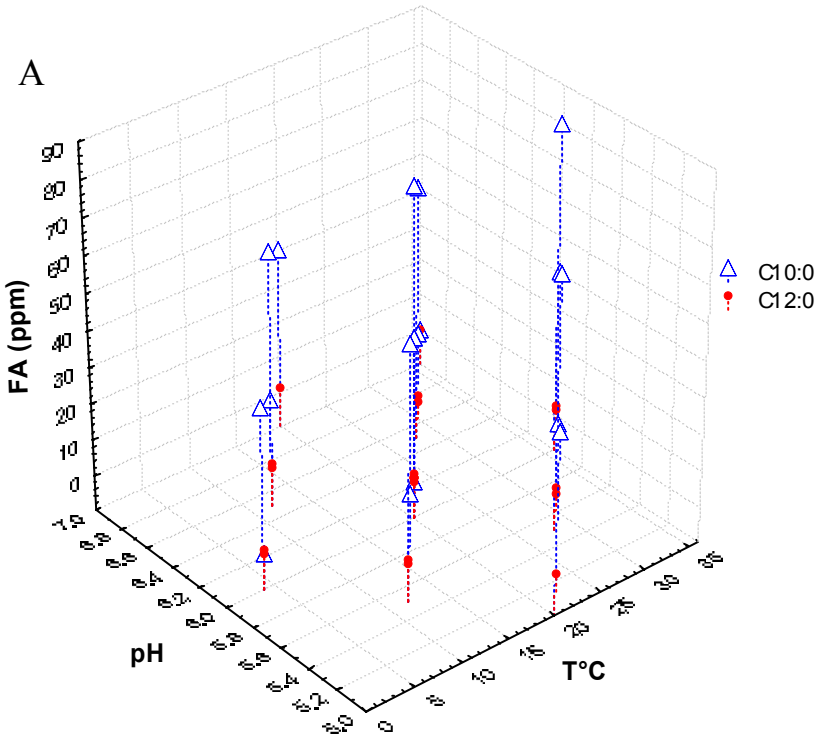
The growth medium consisted of Brain Heart Infusion broth (BHI, from Oxoid, Unipath Ltd. Basingstoke, Hampsire, UK) autoclaved and distributed in quantity of 49 ml in sterile bottles. Fatty acids dissolved in ethanol were then distributed according to the experimental plan in table V-1 and the pH was adjusted using HCl 5M (all purchased from Sigma-Aldrich, St. Louis, MO, USA). Fatty acid stock solutions were prepared in concentrations necessary to obtain the desired quantity

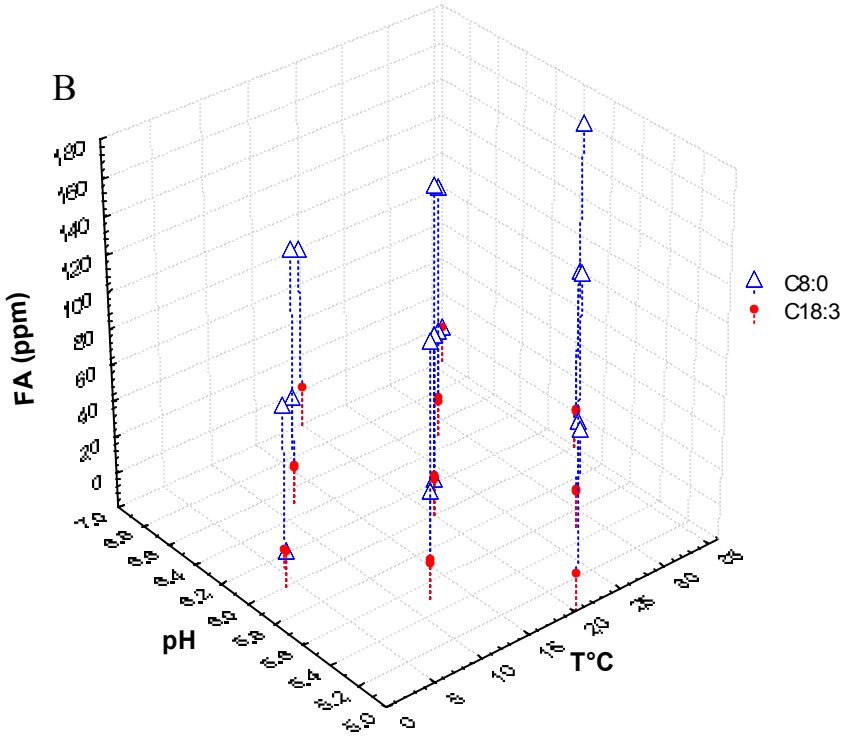
of free fatty acid (FFA) indicated in the experimental plan (table V-1), when dissolving 0.5ml of stock solution in the broth medium. The final ethanol concentration of the growth medium was always 1% V/V.

V-2-1-b Preparation of the inoculum and analysis

Staphylococcus aureus strain ATCC 13565, stored at -80°C was sub-cultured twice in BHI broth at 37°C for 24 hours and used for the analysis. 0.5ml of the appropriate dilution was inoculated in each bottle, in order to obtain a cell concentration of 2-3x10^5 UFC/ml. The inoculated bottles were then incubated at the defined temperatures (table V-1). Every 30 min, the turbidity of samples from all the bottles was measured in a spectrophotometer (UV-Visible spectrophotometer SHIMADDZU, UV-1601, SHIMADZU Italia Srl) at 620 nm. Data was collected until a stability in the OD readings was observed.

Figure V-1: Experimental plan for the effect of non-inhibitory concentration of fatty acids on the growth rate of *S. aureus* under combined conditions of pH and temperature. A)- capric (C10:0) and lauric (C12:0) acids. B)- caprylic (C8:0) and α-linolenic (C18:3) acids





V-2-1-c Turbidity data analysis

The turbidity of the various samples measured over time was transformed into natural logarithm and fitted with the rescaled version of the Baranyi model using the excel add-in DMFit based on Baranyi, J. & Roberts, T.A. (1994) and freely available at http://www.ifr.bbsrc.ac.uk/safety/DMFit/default.html . The 'rescaling' made it possible that the curvature parameters of the fitted sigmoid curves, characterising the transition from the lag to the stationary phase, did not depend on the scale used for the optical density measurements (see the manual of the program). For secondary models, quadratic polynomials were used, for which fitting procedures were also available in the program.

V-2-2 Distribution of the *S. aureus* single cell times to first division and successive generation times

V-2-2a Flow Chamber set up

The flow chamber set up as, described by Elfwing et al., (2004), is the following:

Flow system. The flow system consisted of a feed flask (Schott, Mainz, Germany), autoclavable tubing, a peristaltic pump (P4; Belach Bioteknik, Stockholm, Sweden), a bubble trap manufactured in house, the flow chamber, and a waste flask. The setup is shown in Fig. V-2.

Flow chamber. The flow chamber consisted of five main parts, as shown in Fig. V-3. The device was tightly screwed together with eight steel screws. The cross section was 10 by 0.1 mm, and using a medium volumetric rate of 0.7 ml/min resulted in an average linear velocity of approximately 1.1 cm/s.

Slide preparation. The microscope slides used were either glass slides that were made hydrophobic or polystyrene slides.

(i) DDS coating. An ordinary glass slide (Knittel Gläser, Braunschweig, Germany) was made hydrophobic by dip coating it in 2% dimethyldichlorosilane (DDS) dissolved in 1,1,1-trichloromethane (LKB, Bromma, Sweden). The coated slide was allowed to air dry in a laminar flow hood before it was inserted into a flow chamber.

(ii) Sterile polystyrene slides. Sterile polystyrene cell culture slides (16004; Nalge Nunc International, Naperville, Ill.) were used as delivered.

Flow chamber and tubing preparation. The tubing and flow chamber were autoclaved separately and assembled in a laminar flow cabinet. The setup was connected to the peristaltic pump, and the system was flushed (0.7 ml/min) with sterile phosphate-buffered saline (PBS) (0.2 g of KCl per liter, 0.2 g of KH_2PO_4 per liter, 1.15 g of Na_2PO_4 per liter, 8 g of NaCl per liter; pH 7.3).

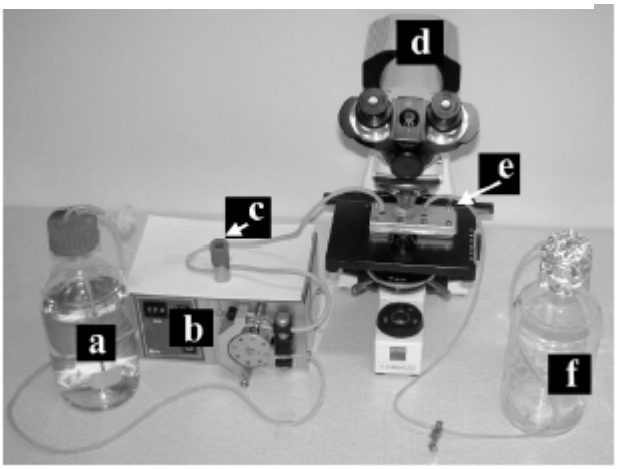
Inoculum. By using a 1-ml syringe and a 0.8-mm-diameter needle, 100 μL of culture was injected into the bubble trap through a rubber membrane. The culture was pumped into the flow chamber, and the bacteria were allowed to settle by switching off the pump. When there was sufficient adhesion (after 15

min), the pump was restarted, and all unattached bacteria were flushed away.

Microscopy and image analysis. The flow chamber was mounted on the stage of a dark-field microscope (Zeiss Standard 25; Zeiss, Oberkochen, Germany) equipped with either a X4 objective

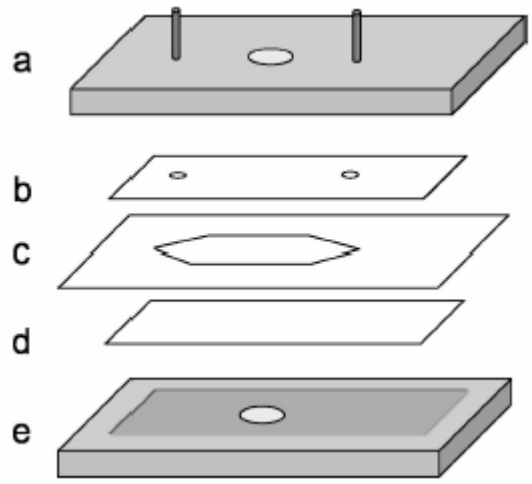
(Leiz, Wetzlar, Germany) or a X10 objective (Zeiss). A high-resolution (1040 by 1392 pixels) charge-coupled device camera (CoolSnap Pro cf; Roper Scientific, Trenton, N.J.) was mounted on the microscope, and the digital signal was transferred to a PCI interface card. An image analysis program (Image Pro Plus; Media Cybernetics, Silver Spring, Md.) controlled the camera to grab an image every 1 to 5 min depending on the required frequency. The program could semi automatically improve the image quality by using built-in functions to remove background noise and enhance object edges. Image Pro Plus was able to automatically recognize clusters of brighter pixels as cells and to calculate the area of every cell and designate every cell with *x* and *y* coordinates. An in-house Visual Basic program transferred the size (in pixels) and the *x* and *y* coordinates to a spreadsheet, where the data were analyzed further. The images were grabbed at a low magnification (X200) by using dark- field illumination. The area of the computer image was 1,657 by 1,238 μm, as measured with an objective micrometer scale (Zeiss). This area was large enough to include as many as 1,500 to 3,000 bacteria. Although the low magnification resulted in very few pixels (20 to 30 pixels) per cell, the resolution was sufficient for our purposes since we were only interested in observing the division times of the cells. Note that, as Lawrence et al. (1989) pointed out, it is impractical to try to estimate the size of a cell (in micrometers) based on dark-field images, since the size is inflated three- to sevenfold.

Figure V-2: flow chamber system set up (Elfwing et al., 2004)



a, feed flask; b, peristaltic pump; c, bubble trap; d, microscope equipped with a charge-coupled device camera; e, flow chamber; f, waste flask.

Figure V-3: Flow chamber set up (Elfwing et al., 2004)



(a) Top block of aluminium with inlet and outlet pipes (diameter, 1.2 mm) with two O rings; (b) polycarbonate slide with two holes (diameter, 1.2 mm); (c) polymer spacer; (d) microscope slide; (e) bottom block of aluminium.

V-2-2-a Inoculum preparation

Staphylococcus aureus strain ATCC 13565, stored at -80°C was sub-cultured twice in BHI broth at 37°C for 24 hours before being used for the analysis. 100 µL of the preinoculum was inoculated in the bubble trap and carried to the flow chamber with the flow of PBS. After a while, the tube connecting the waste flask and the flow chamber was closed as well as the pump, in order to let the cells attach to the microscope slide.

V-2-2-b Preparation of fatty acids stock solutions and growth medium

Fatty acid stock solutions were prepared as indicated previously, in order to have a final concentration of ethanol in the medium of 1% v/v and the concentration of fatty acids indicated in Table V-2. The pH was adjusted using HCl 5M. BHI broth was prepared and sterilized using disposable 20µm filtering bottles connected to a vacuum pump, and successively added of the necessary quantity of fatty acid solution. PBS was prepared as indicated previously and also sterilized by filtration.

V-2-2-c single cells growth and data collection

After cells have attached, the PBS flow was left closed and the BHI modified according to the experimental plan (table V-2) was let to flow in the chamber. With the reopening of the flow, the non attached cells were flushed out and the attached ones were considered to have begun the growth. Microscopic analysis was performed as indicated previously, and cell images were capture every 5 min and recorded. Using an IFR (Institute of Food Research Norwich Research Park, Colney, Norwich NR4 7UA, UK) in-house program, the size of each cell (in pixels) detected under the microscope was recorded over time. The times of divisions were measured by the sudden drops in pixel sizes, attributing this fact to the removal of the daughter cells immediately after the division. These times of division were recorded using and IFR in-house excel add-in written in Visual Basic. Physiologically, the time to the first division is the sum of the lag time and the first generation time of the observed cell, but for the sake of simplicity we will speak about the 'time to the first division' as the lag time of single cells. The subsequent generation times were defined as the intervals between two successive divisions: ith generation time = time to the ith division – time to the (i-1)th division (i>1) (Pin and Baranyi, 2006).

Table V-1: Experimental design for single cell analysis

	Fatty acids tested			
pH	Control	C18:3	C12:0	C10:0
Incubation	37°C			
pH7	0 ppm	1ppm	1ppm	20ppm
pH5	0ppm	1ppm	1ppm	20ppm

V-2-2-d Simulating the growth of the population from the distribution of the lag time of single cells

The measured distributions (histograms) of the time to first second and successive division times were used to simulate the population growth curves as proposed by Pin and Baranyi (2006). Their model is based on the following assumption:(i) The first division is the sum of the lag time and the first generation time. (ii) After the *k*th division, the daughter cells are in exponential growth phase, having the same distribution for their generation times as for the last (*k*th) observed one. (iii) The distributions of the second and successive generation times (up to the *k*th) are those measured by image analysis. The value of *k* is usually around 3 or 4 (Pin and Baranyi, 2006). This means that we consider the cells uniformly in the exponential phase only after 3 or 4 divisions; until then the distribution of the generation times gradually converge to their distribution in the exponential phase.

Details of the algorithm used for the simulation is available in Pin and Baranyi, (2006) and was implemented in an IFR in-house Excel add-in program written in Visual Basic. 20 simulations were performed for all the studied inoculum sizes (2, 10, 100, 800 cells) and observed experimental distributions. The estimation of the population growth parameters (maximum specific growth rate and lag time) were done by fitting the model of Baranyi and Roberts, (1994) to the growth curves. The distribution were compared using the Barlett test of homogeneity implemented in an IFR in-house Excel add-in program VariFit (BACANOVA) project.

V-2-2-e Plate count Growth curves of cell populations measured by plate counts

Bottles containing 100ml of BHI broth adjusted to pH 7 were prepared as indicated in table V-2. *S. aureus* preinoculum prepared as indicated previously was diluted in BHI broth pH7 and inoculated in order to obtain 800 cells per 100ml of growth medium. The bottles were then incubated in a water bath at 37°C and at each sampling time, the cell load was estimated by plating on BHI agar. The comparison of the simulated and the observed growth curve parameters were carried out by using F-tests (Brown and Rothery: Models in Biology; Wiley, 1993) implemented on and Excel working sheet (IFR in-house use) .

V-3 Results

V-3-1 Influence of fatty acids (FAs), temperature and pH on the specific growth rate of *S. aureus*

In order to assess the influence of non-inhibitory concentration of α-linolenic, lauric, capric and caprylic acid, pH and temperature on the specific growth rate of *Staphylococcus aureus*, the experimental plan in figures V-1A and V-1B was designed. The level of fatty acids were chosen at concentration lower than half of the smallest minimal inhibitory concentration observed in the precedent experiment. This choice was done in order to avoid any risk of partial inactivation of the population during the experiment. In table V-2, coefficients of the secondary model equations, their respective standard errors, the model coefficients and the root mean square errors are reported. As it can be observed, the best fitting models describing the effect of the free fatty acids, temperature and pH did not include the same terms, indicating different interactions between the three factors.

Table V-2: Coefficients of the generated quadratic polynomial models describing the OD-rates of *S. aureus*. as functions of free fatty acids, temperature and pH.

variables	C18:3	se	C12:0	se	C10:0	se	C8:0	se
const	0.86315	0.28644	1.1604	0.32634			-4.2793	1.9466
T	-0.05763	0.01262	-0.07154	0.014043	-0.03366	0.01222	-0.04625	0.030304
pH	-0.14158	0.04788	-0.20611	0.054231			1.4989	0.64267
[FA]								
T*pH	0.00915	0.00210	0.01338	0.002283	0.00603	0.00197	0.011156	0.004868
T*[FA]	-0.00128	0.00042			-0.00011	0.00004	-4.92E-05	1.65E-05
pH*[FA]			-0.00281	0.001805				
T^2	0.00032	0.00007			0.00042	0.00012		
pH^2							-0.13398	0.054518
R^2	0.93		0.916		0.82		0.874	
RMSE	0.032		0.038		0.094		0.074	

T, (temperature); [FA] fatty acid concentration; se (standard error); RMSE (root mean squared error)

In figures V-4a, V-5a, V-6a and V-7a, 3D-plots representing, at pH=6, the OD growth rates as functions of temperature and octanoic, decanoic, lauric and α-linolenic acids, respectively, are reported. At refrigerated temperatures, the FAs tested did not cause any sensible reduction of the OD-growth rate as their concentration increased; even though C8:0 and C10:0 were more active than C12:0 and C18:3. On the other hand, the fatty acid inhibition potential increased with the temperature. Figures V-4b, V-5b, V-6b, V-7b take into consideration the effect of pH and FAs at temperature 20°C on the growth rate. In presence of C8:0 and C10:0, the specific growth rate of *S. aureus* showed a plateau when the pH was higher than 6.5, due to the presence of quadratic terms of the pH in the model. In general the trend in the inhibition of the specific growth rate was the same for all the fatty acids, and in particular C8:0 was the FA less active while C18:3 the most inhibitory. At low concentrations (lower than the minimal inhibitory concentration), fatty acid influence on the specific growth rate was very little, specially at low temperatures, and the inhibitory potential of the FAs was stimulated more by the temperature than by the pH.

Figure V-4: Influence of octanoic acid on the OD growth rate of S. aureus; (a) as function of temperature at pH6, (b) as function of pH at temperature 20°C.

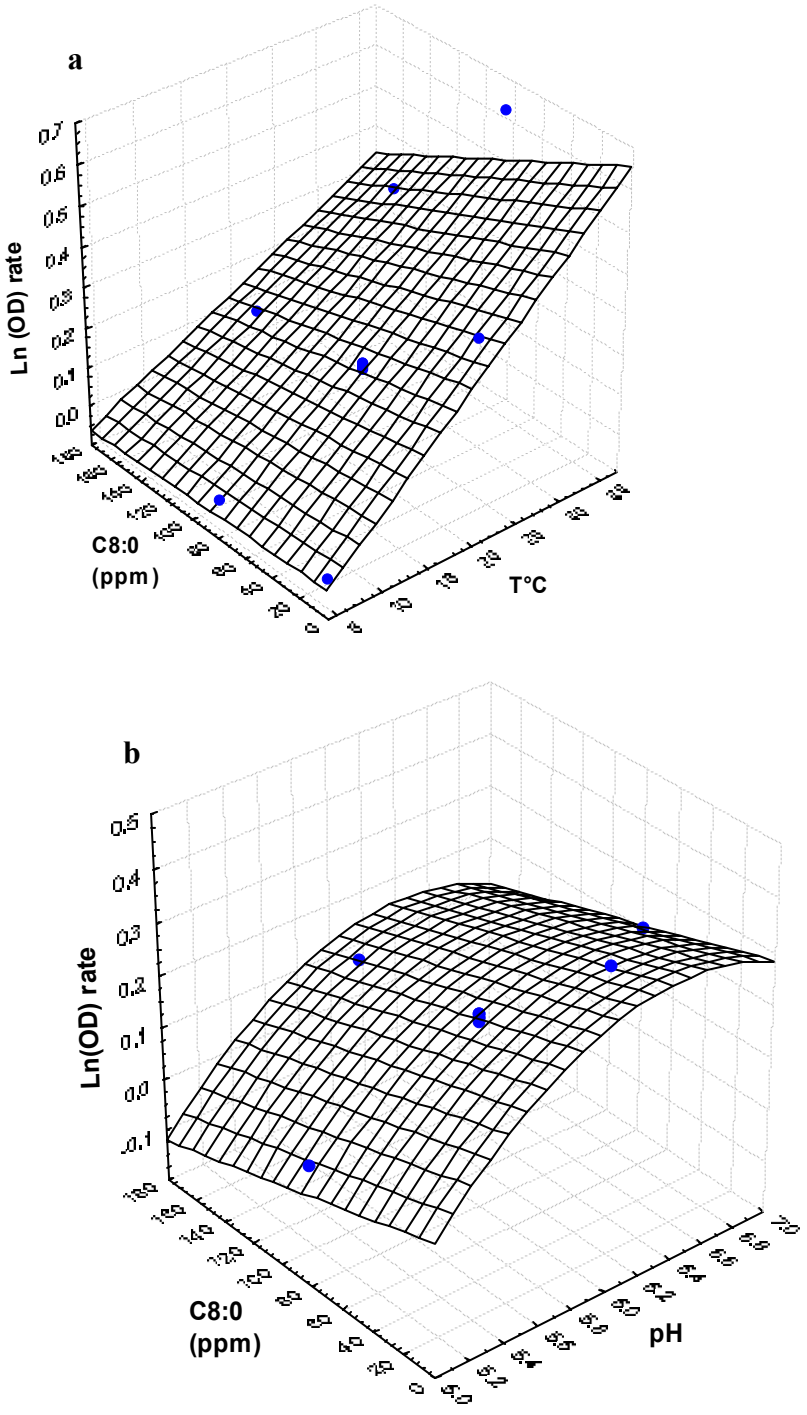


Figure V-5: Influence of decanoic acid on the OD growth rate of S. aureus; (a) as function of temperature at pH6, (b) as function of pH at temperature 20°C.

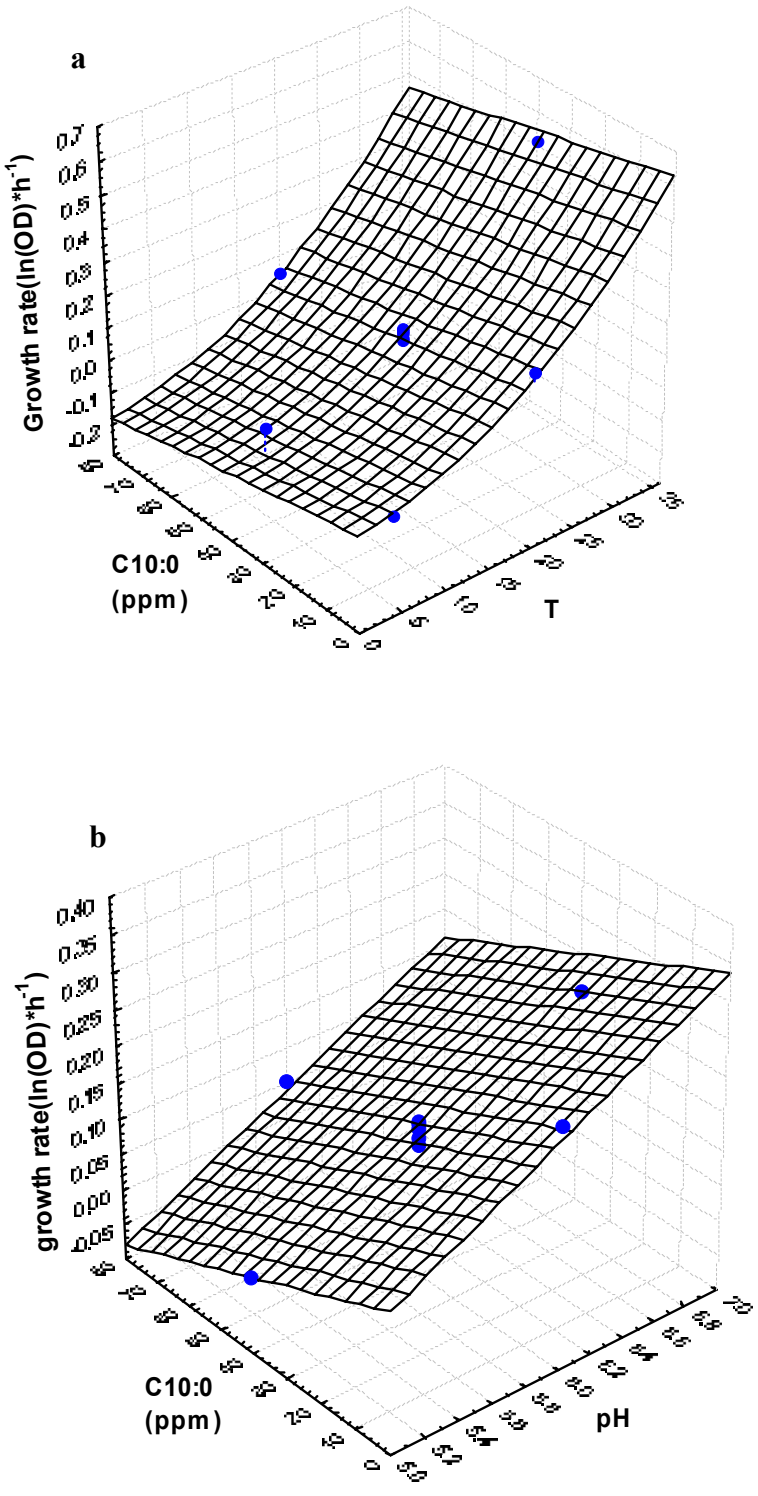


Figure V-6: Influence of lauric acid on the OD growth rate of S. aureus; (a) as function of temperature at pH6, (b) as function of pH at temperature 20°C.

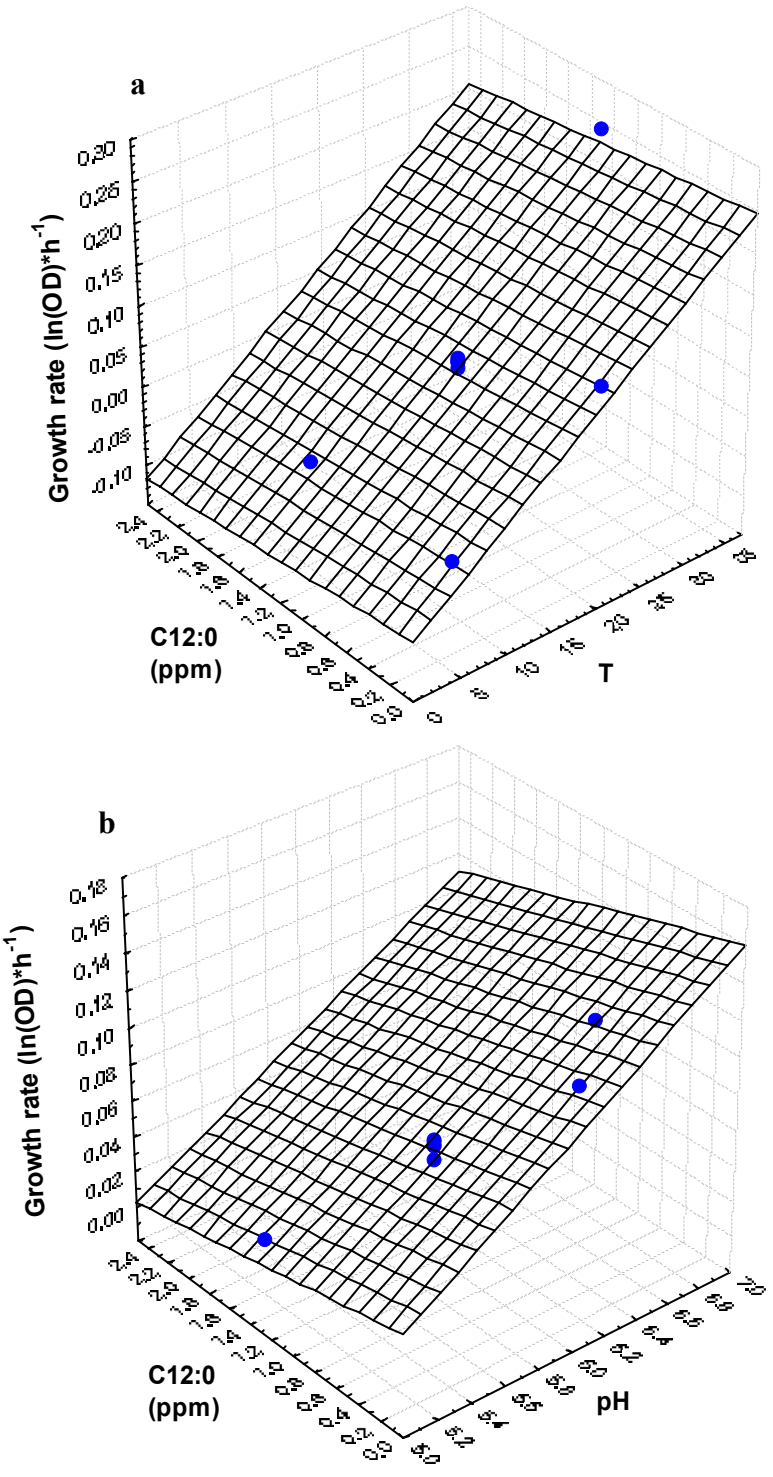
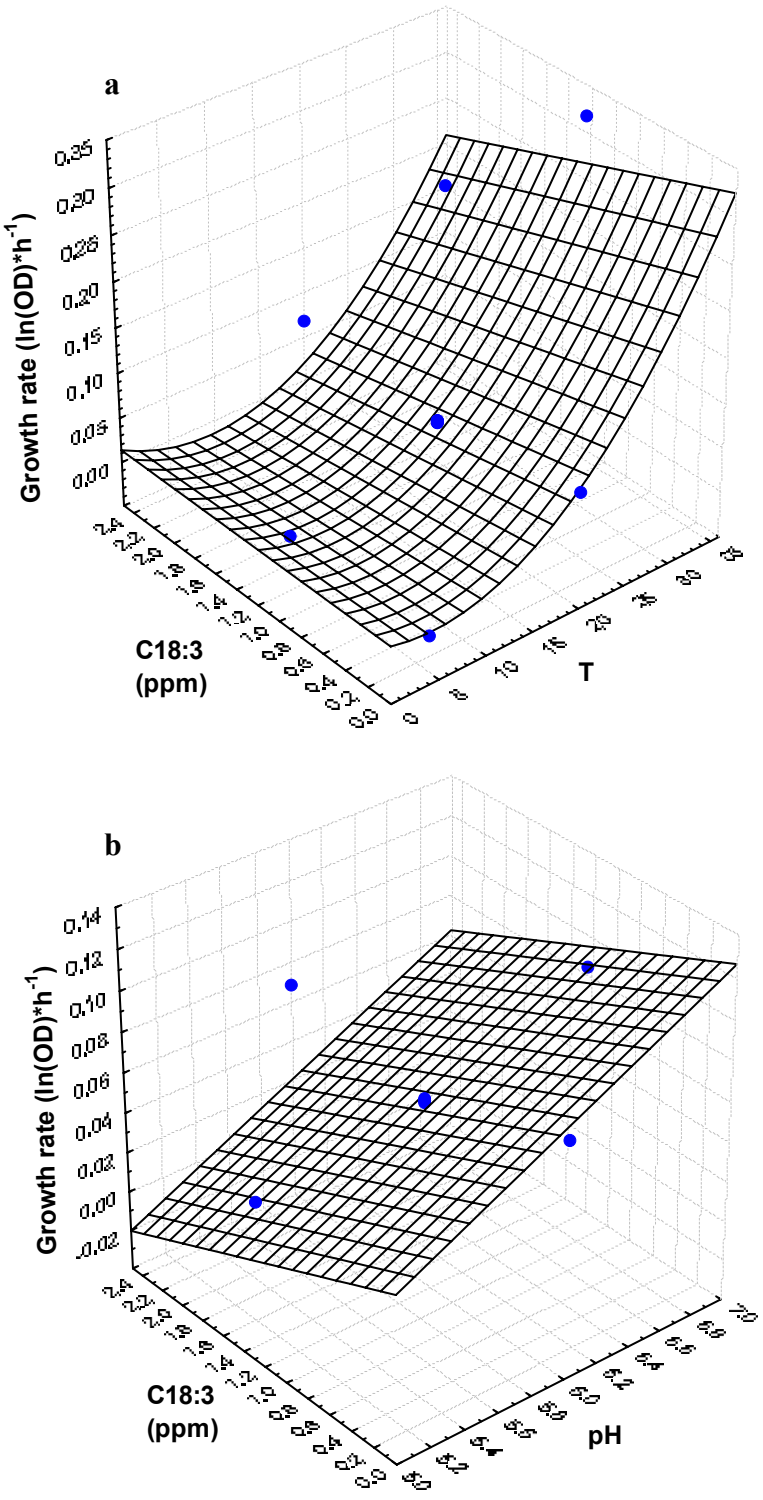


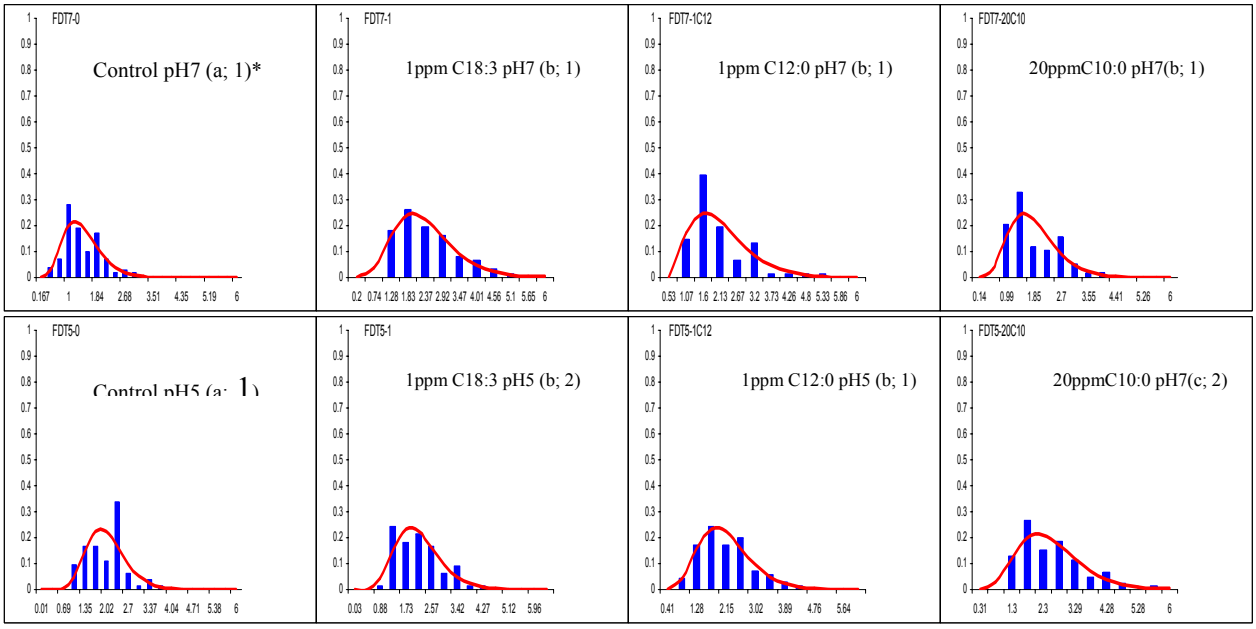
Figure V-7: Influence of α-linolenic acid on the OD growth rate of S. aureus; (a) as function of temperature at pH6, (b) as function of pH at temperature 20°C.



V-3-2 Single cell lag time and second generation time (SGT) of *S. aureus* in presence of free fatty acids (FFAs).

The influence of selected fatty acids on the lag (as said, identified by the 'time to the first division', though physiologically that is rather the sum of the lag and the first generation time) and the subsequent generation times of *S. aureus* was assessed using the experimental design in table V-2. The fatty acid concentrations tested were chosen in order to represent more or less the same proportionality with respect to the MIC observed previously. During the experiment, consistent data were recorded only for the lag and the second generation time. This difficulty was due to the biofilm formation of the *S. aureus* strain used, which at a certain point of the experiment caused the clouding of the microscope slide and also disturbed the normal detachment of daughter cells and their flushing out of the chamber. In figures V-8 and V-9, the respective observed distributions of the lag and SGT of *S. aureus* in the presence of FFAs are shown.

Figure V-8: Barlett test of homogeneity of the variances of the time to first division distributions of *S. aureus* single cells grown at pH 7 and pH5, in the presence of α-linolenic acid (C18:3), lauric (C12) and capric (C10) acids at the concentrations of 1, 1 and 20ppm respectively

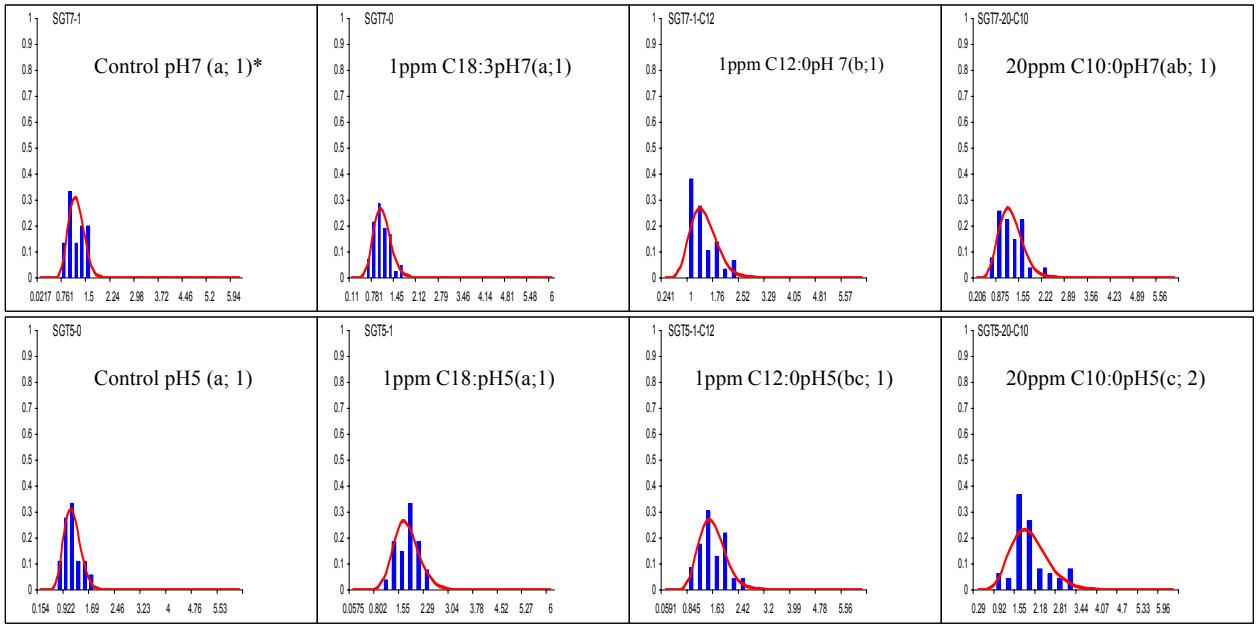


*In bracket, the same letters in the same row means that the distributions are homogeneous, and the same number in the same column means that the distributions are homogeneous at a 5% uncertainty

At pH7, the distribution of TFD when the cells were in the presence of C18:3, C12:0 and C10:0 at the concentration tested, were different to that of the control according to the Barlett test of

homogeneity. On the other hand, when the pH was brought to 5, the distribution of the TFD when in presence of C18:3 and C12:0 were homogeneous but different to that of *S. aureus* in the control test and in the presence of C10:0, this latter different from all.

Figure V-9: Barlett test of homogeneity of the variances of the second generation time distributions of *S. aureus* single cells grown at pH7 and pH5 in the presence of α-linolenic acid (C18:3), lauric (C12) and capric (C10) acids at the concentrations of 1, 1 and 20ppm respectively



*In bracket, the same letters in the same row means that the distributions are homogeneous, and the same number in the same column means that the distributions are homogeneous at a 5% uncertainty

Only in the control experiment and in the presence of C12:0, the *lag* distribution of *S. aureus* single cells at pH 7 and at pH 5 resulted homogeneous while the variance of the distributions in the presence of C10:0 and C18:3 were statistically different as pH changed. Regarding the second generation time, at pH 7, distributions were homogeneous with that of the control when the cells were in presence of C18:3 and C10:0, while in the presence of C12:0, the distribution was different from that of the other experimental conditions (fig. V-9). At pH5, differences between the SGT distributions were observed only between C12:0 or C10:0 and that of *S. aureus* in the presence of C18:3 and the control. In general, the SGT distributions were less scattered than the TFD.

To further evaluate the differences between the distributions, their means and standard deviations were compared (table V-3 and V-4). At pH 7, the mean of the TFD, when *S. aureus* single cells were in contact of FFAs, were statistically different from that of the control. On the other hand at pH 5, the means of the TFD distributions when the cells were in the presence of C18:3

and C12:0, were equal to the control and significantly lower than that observed when the cells were in the presence of C10:0. When testing the same FFA at pH 7 and pH 5, differences of the TFD distribution mean were observed only with the control and C10:0 (table V-3). Concerning distribution of the second generation time, the means at pH 7 and pH 5 in presence of FFAs were higher than that of the control, while between the FAs no difference was observed (table V-4). Within the same fatty acid experimental conditions, the SGT distribution mean was different when changing the pH from 7 to 5 only when C18:3 and C10:0 were tested on *S. aureus*.

Table V-3: Mean and Standard deviation of the first division time (FDT) of *S. aureus* single cells as influenced by α-linolenic acid (C18:3), lauric (C12) and capric (C10) acids at the concentrations of 1, 1 and 20ppm respectively

BHI Mean FDT	Control 1% ethanol 96%	1 ppm C18:3 1% ethanol 96%	1ppm C12 1% ethanol 96%	20ppm C10 1% ethanol 96%
PH7	$1.28\pm0.56^{a;1}$	$2.1\pm0.95^{c;1}$	$1.85\pm0.93^{bc;1}$	$1.63\pm0.75^{b;1}$
PH5	$1.86\pm0.56^{a;2}$	$1.94\pm0.74^{ba;1}$	$1.93\pm0.76^{ba;1}$	$2.29\pm0.99^{c;2}$

Table V-4: Mean and Standard deviation of the second generation time (SGT) of *S. aureus* single cells as influenced by α-linolenic acid (C18:3), lauric (C12) and capric (C10) acids at the concentrations of 1, 1 and 20ppm respectively

BHI Mean SGT	Control 1% ethanol 96%	1 ppm C18:3 1% ethanol 96%	1ppm C12 1% ethanol 96%	20ppm C10 1% ethanol 96%
PH7	$0.95\pm0.25^{a;1}$	$1.04\pm0.23^{ab;1}$	$1.26\pm0.38^{b;1}$	$1.09\pm0.33^{b;1}$
PH5	$1.02\pm0.24^{a;1}$	$1.55\pm0.37^{bc;2}$	$1.37\pm0.39^{b;1}$	$1.71\pm0.55^{c;2}$

V-3-3 growth curves simulations from the observed single cells distributions

The observed single cell lag and SGT distributions were used to simulate growth curves from different initial cells using the model and algorithm of Pin and Baranyi (2006). In order to validate the results of the simulation studies, growth curves of *S. aureus* with initial inoculum of 818 cells were obtained with plate count data, performing the experiment in the same conditions as carried out with the flow chamber at pH 7. The growth curves obtained were fitted to the Baranyi model and the growth parameters *lag* time compared to those of the simulated growth curves.

Table V-5: Comparison of simulated and observed *lag* times obtained from an inoculum of 818 cells.

Growth parameter	Simulated Mean ± se	Observed Mean ± se	F	P	differences between Simulated and observed
lag-control	0.619±0.025	0.808±0.198	1.366	0.253	NS
lag-C18:0	1.059±0.034	0.995±0.521	1.688	0.205	NS
lag-C12:0	0.767±0.038	0.896±0.065	0.326	0.573	NS
lag-C10:0	0.571±0.03	0.68±0.083	1.938	0.175	NS

In table V-5 the estimated *lag* times of the simulated and observed growth curves for the same initial cell inoculum of 818 cells, as well as the results of their comparison, are reported. No significant difference was observed comparing data of observed growth curves *lag* times with those obtained from curves generated from the single cell TFD and SGT distribution. Growth rates were not taken into consideration for the insufficient observed generation times. These results, legitimate further considerations regarding the lag times, on data obtained by means of simulations. Moreover the approach gives us the possibility to study cases of very low initial cell load, difficult to perform in laboratory for evident reasons. Twenty growth curves were then generated from every set of distributions (TFD + SGT) and for different initial cell loads (818, 100, 10 and 2), in order to evaluate the influence of inoculum, FFAs and pH on the *lag* time and *lag* time variability. Figures V-10a and V-10b show respectively the mean *lag* times and standard deviation (SD) of the generated growth curves obtained form cells grown at pH 7. Fatty acid effects on the lag were of different degree of magnitude, confirming their different antimicrobial potentiality. In particular, C18:3 was the FFA that most prolonged the lag phase of *S. aureus*, while C10:0, even if at 20ppm

did not influence the *lag* with respect to the control experiment (fig V-10a). Moreover, in the control experiment and in the presence of C12:0 and C10:0, at pH 7, the *lag* time increased in absolute value with the initial cell load reduction. When evaluating the standard deviation associated to the different *lag* time means (fig V-10b), it can be deduced that in presence of C18:3 and C10:0 the *lag* times were more scattered than when *S. aureus* was in the control or in presence of C10:0. Figure V-10b, also enlighten the increase of the *lag* time variability with decreasing initial cell load numbers.

Figure V-10a: Mean *lag* times from 20 growth curves generated from the TFD and SGT of *S. aureus* at pH 7 in presence of selected FFAs.

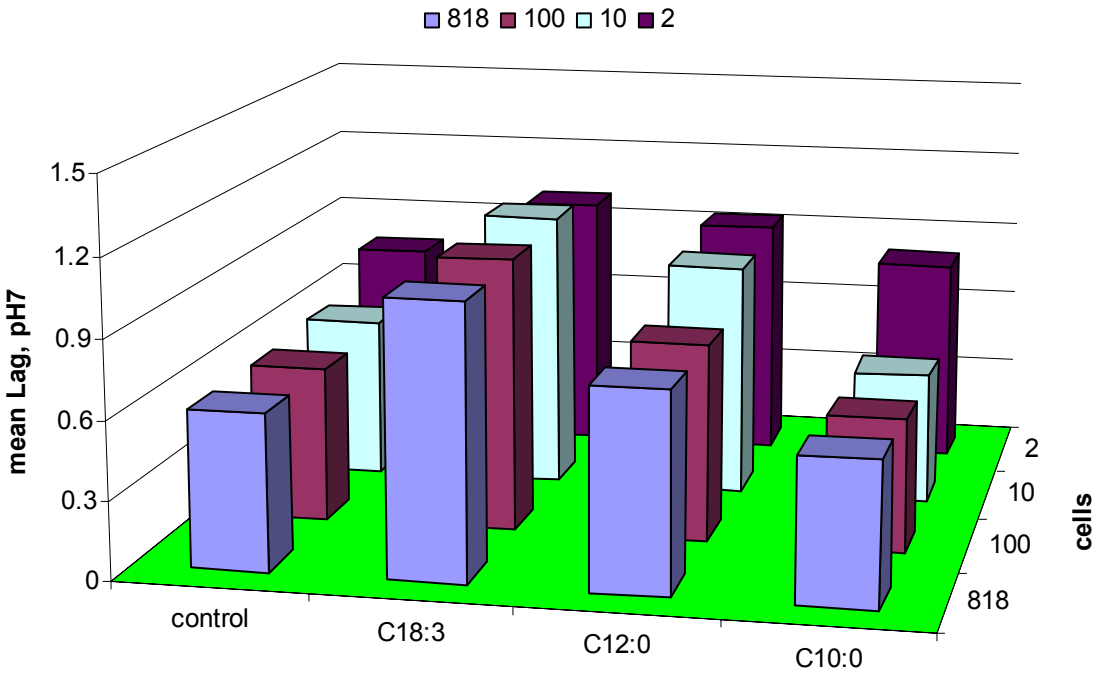


Figure V-10b: Mean *lag* times standard deviations (SD)from 20 growth curves generated from the TFD and SGT of *S. aureus* at pH 7 in presence of selected FFAs.

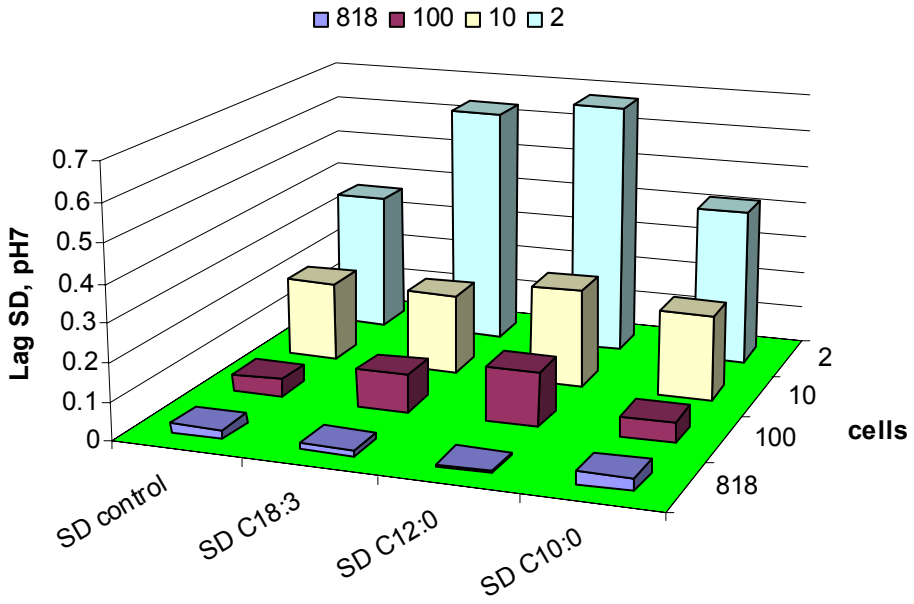


Figure V-11a: Mean *lag* times from 20 growth curves generated from the TFD and SGT of *S. aureus* at pH 5 in presence of selected FFAs

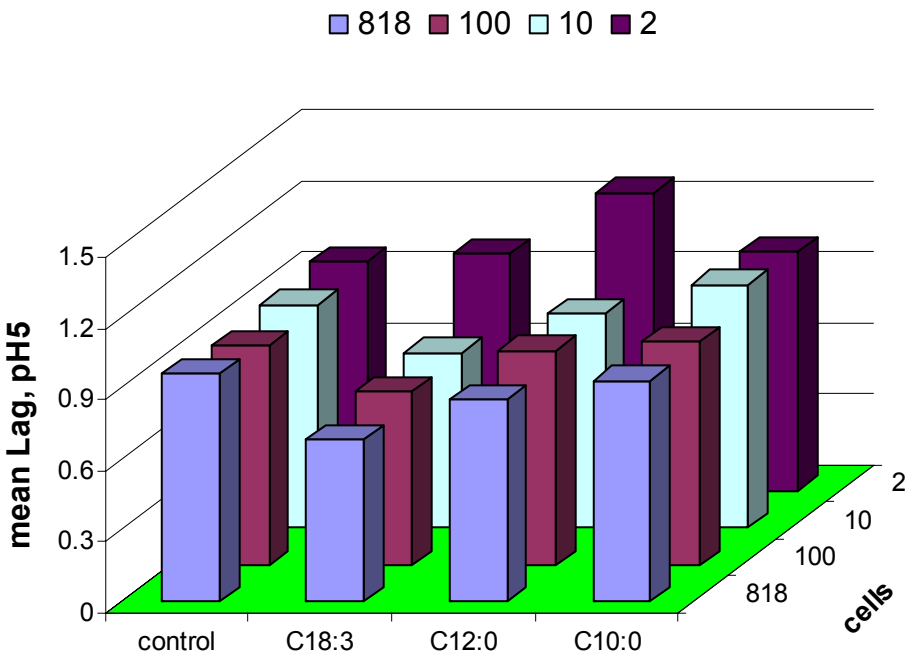
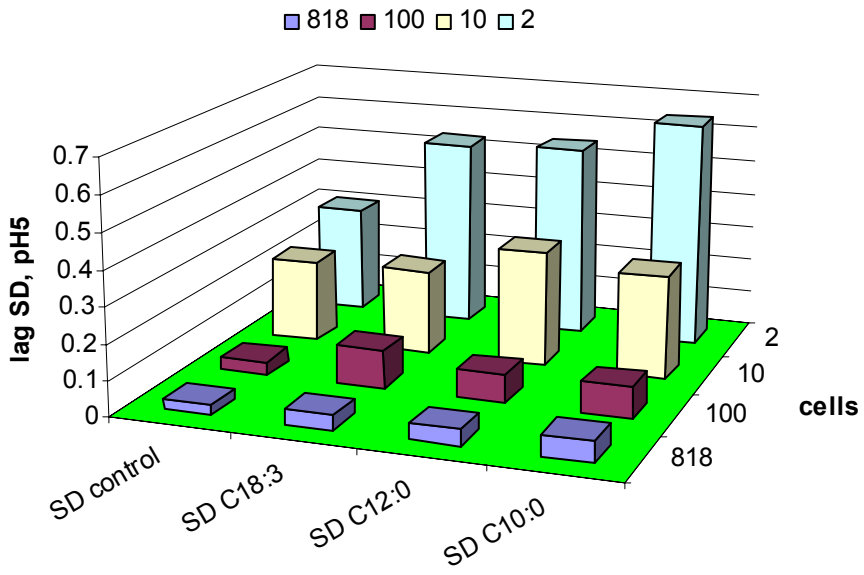


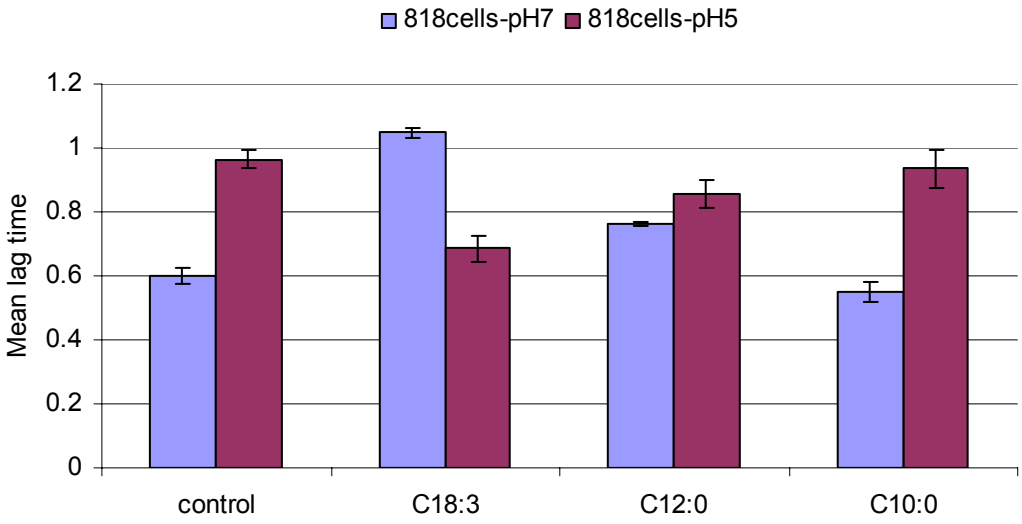
Figure V-11b: Mean *lag* times standard deviations (SD)from 20 growth curves generated from the TFD and SGT of *S. aureus* at pH 5 in presence of selected FFAs



At pH5, the effects of the selected fatty acids on the mean *lag* times and standard deviations (figure V-10a and V11b respectively) showed the same trend discussed previously at pH 7. The only

difference resulted in the fact that mean *lag* time when *S. aureus* was exposed to C18:3 increased with the reduction of cell load whereas it was quite constant at pH 7. Regarding the SD, it increased with the reduction of initial cell load, the fatty acid chain length and inhibition potential (observed in previous experiments). The pH also showed to have an influence on the *lag* time. In general, the reduction of the pH from 7 to 5 induced an increase in the lag time when *S. aureus* was exposed to C12:0 and C10:0 and in the control experiment. In the presence of C18:3, *S. aureus* showed and inverse trend, having higher *lag* times at pH 7 than pH 5, aspect clearly observed in figure V-12.

Figure V-12: Mean *lag* times of *S. aureus* from an initial inoculum of 818 cells, generated from the observed single cells TFD and SGT distributions, at pH 7 and pH 5.



V-4 Discussion

Free fatty acids (FFAs) are present in quite all foods we eat. Their concentration during the storage can increase or reduce as the effect of microbial or food enzymes activities. The antimicrobial activity of many free fatty acids has been studied mostly at physiological temperature and pH (Kabara et al., 1972; Lee et al., 2002), conditions difficult to find in food environment. It is hence necessary to evaluate the antimicrobial potentialities of these FFAs combining as many factors related to food as possible. Another important fact is the study of antimicrobials at concentrations

really available in food. Under the minimal inhibitory concentration, antimicrobial compounds perform their activity most likely on the microrganism lag phase and the growth rate (i.e. kinetic parameters).

In this work an experiment was done an analysed to assess the combined effect of non inhibitory concentration of FFAs, pH and T°C on the specific growth rate of *S. aureus*. According to our data, at low concentrations, the fatty acids did not showed any considerable inhibition of the growth rates. Moreover, the pH did not enhance the FFAs inhibition potentials while the temperature increased the activity only of C10:0 and C8:0. This result is probably related to the physical properties of the FFAs . In fact, the increase in solubility and hence of the availability is very high for C10:0 and C8:0 when the temperature increased with respect to C12:0 and C18:3 (Sado et al., 2006). The absence of an important synergic effect between pH and FFA in the models indicates that the dissociated/undissociated state did not have any effect on specific growth rate. In fact the pH and temperature generally affect the activity of weak organic acid by influencing their undissociated and dissociated from ratio (Le Marc et al., 2002). Fatty acid action on microrganism growth is generally correlated to their capacity to diffuse through the cell membrane affecting its functionality and/or the inhibition of the fatty acid biosynthesis (Bergsson et al., 2001; Zheng et al., 2005). The low influence of FFAs at the concentration tested on *S. aureus* growth rate also indicates that the division times of subsequent generations do not have a high dependency on FFA concentration.

The experiment carried out with the flow chamber to measure the single cell TFA and SGT indicates that the lag phase is the growth phase most affected by the fatty acids tested. The effect of the fatty acids were more pronounced on the TFA distribution than on the SGT distribution. This means that in presence of fatty acids, there was a different distribution with a shift of the mean towards high values. Métris et al (2003) found that the more inhibitory is the growth condition , the longer is the detection time and the more scattered is their distribution. Based on the fact that the detection time can be considered as a delayed lag time, the findings of Métris et al., (2003) are also true in this experiment. In fact, both the mean and the standard deviation of the distribution were higher in the presence of FFAs compared to the control experiment. After the TFD distribution, the SGT were less scattered, indicating that *S. aureus* cells response became more homogeneous after overcoming the task necessary for the first division.

The simulated growth curve *lag* times showed no difference with respect to those of the plate count growth curves, while it could not be the case for the specific growth rate. In fact at least 3 to 4

generation times are necessary for a microrganism to arrive at its maximum growth rate (Métris et al., 2005). So, in order to generate growth rates from the distributions, at least 3 to 4 generation times are needed because cells do not enter immediately in the exponential phase after the TFD (Pin and Baranyi, 2006; Metris et al., 2005). This is the reason why with our data we could not generate the specific growth rate. These results demonstrate that the measured TFD and SGT are enough to generate the population lag times using the stochastic model and algorithm proposed by Pin and Baranyi, (2006). An effect of the inoculum size on the lag respect to that of the population was observed only when the initial cells were equal or lower than 10. Theses results, very important in terms of risk assessment, emphasize the difficulty of predicting the *lag* time, due to the high variability correlated to this parameter, when in presence of very low number of cells. In presence of C18:3 and C12:0, the most inhibitory FFAs among those tested, a higher lag time variability was observed. This variability seams not to be correlated to the mechanism of inhibition of the fatty acids. In fact the most accepted mechanism for C18:3 is the activity at the cell fatty acids biosynthesis pathway (Zheng et al., 2005), while for C12:0 an other fatty acids, the disruption of the cell membrane is the proposed mechanism (Bergson et al., 2001). Respect to medium chain fatty acids, the *lag* time of C18:3 was higher at pH 7 than at pH 5, suggesting a different mode of action of the two groups. In fact, if C18:3 inhibits the enzyme FabI responsible of the fatty acid elongation during FA synthesis, as a protein, this will react better with the substrate (C18:3) at pH 7 than at pH 5.

V-4 Conclusion

Fatty acids, at concentration lower than the minimal inhibitory concentration influence more the lag phase of *S. aureus* than the specific growth rate. The pH affects differently the inhibition potential of C18:3 on the *lag* time of *S. aureus*, respect to C12:0 and C8:0. Moreover, the variability of the lag increases in the presence of FFAs and with the reduction of the initial inoculum. The use of the flow chamber microscopy images to measure the individual cells division times has demonstrated to be a useful tool to assess the influence of antimicrobial like fatty acids on the bacterial growth parameters at low inoculum concentrations. Further research need to be done on this topic to help in the understanding of the effect of antimicrobial, specially on low cells concentrations.

V-5 References chapter V

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CHAPTER VI

FREE FATTY ACIDS INTERACTION WITH *Staphylococcus aureus*: EXTRACELLULAR RELEASE OF ESTERASE AND *de novo* FATTY ACIDS BIOSYNTHESIS

VI-1 Introduction

Exogenous free fatty acids (FFAs) can interact with bacteria by influencing their growth dynamics. This influence can lead to the death, delay or stimulation of microbial growth. The mechanism by which FFAs affect cellular functions is still not totally understood. So far, according to the literature, the activity of exogenous fatty acids seems to be related to their physical properties and level of involvement with the bacterial metabolism (Kabara et al., 1972; Lee et al., 2002; Zheng et al., 2005). Although most bacteria lack polyunsaturated fatty acids (PUFA), it is known that the majority of bacteria can take up exogenous PUFA present in the culture medium (Watanabe et al., 1994). In fact tween 80 is used as a source of oleic acid for the preparation of media for the growth of lactic acid bacteria. Some bacteria, mainly marine bacteria, even possess the metabolic capacity to synthesize PUFA (Russell et al., 1999). Kankaanpää et al. (2004) investigated whether specific probiotic strains could incorporate exogenous free PUFA into cellular fatty acids and how these changes could influence the physical properties of the bacteria. The results indicated that lactobacilli do incorporate exogenous free PUFA into their cell lipids and that free PUFA in the growth medium of lactobacilli may induce changes in fatty acids composition in relation to the regulation of the degree of fatty acid unsaturation, cyclization and proportions of PUFA containing 20 to 22 carbons and 18 carbons with conjugated double bonds. Despite these changes in cellular fatty acids, only minor alterations in the electron donor-electron acceptor properties of the lactobacilli were observed, suggesting that exogenous PUFA did not adhere to cell surfaces during their harvesting but were assimilated by the lactobacilli. As free PUFA have been shown to be antibacterial compounds (Kankaanpää et al., 2001), the demonstrated PUFA assimilation may indeed be a detoxification mechanism used by lactobacilli (Jiang et al., 1998). Also, the growth of some lactic acid bacteria in medium with the presence of oleic acid can enhance their resistance to low acid environments

(Corcoran et al., 2007). During their growth, some bacteria as staphylococci perform lipolytic activities with the release of free fatty acids by the means of lipases and esterases. Casaburi et al. (2006) found that the esterase activity of some strains belonging to different species of staphylococci is probably due to intracellular or cell-enveloped associated enzymes, because the esterase activity was higher when using whole cells and cell extracts than the extracellular extracts. This is an indication that any disrupting action in the cell membrane can lead to an increase in esterase enzymes in the extracellular medium. If the mechanism of action of an antimicrobial compound is directed towards the cell cytoplasmic membrane, then its activity can be indirectly measured by the quantity of esterases released in the growth medium.

The variation in cell lipid composition depends on the extracellular fatty acids up take or on their *de novo* biosynthesis. Fatty acid biosynthesis, which is the first stage in membrane lipid biogenesis, is catalysed in most bacteria by a series of small soluble proteins that are each encoded by a discrete gene. This arrangement is termed the type II fatty acid synthase (FAS) system and contrast sharply with the type I FAS of eukaryotes which is a dimmer of a single large, multifunctional polypeptide (Heath et al., 2001). The fatty acid biosynthesis pathway offers several unique sites for the selective inhibition by antibacterial agents. Fatty acid biosynthesis is co-ordinately regulated with phospholipids synthesis, macromolecular synthesis and growth as part of the normal response of a bacterium to a changing environment. Gram-positive bacteria do not make unsaturated fatty acids by *de novo* synthesis, and thus do not contain homologs of FabA or FabB (Heath et al., 2001), but when these fatty acids are present, they are synthesized from existing saturated acyl chains via an inducible desaturase (Aguilar et al., 1998). The FabI that has a *tran*-2-Enoyl-ACP reductaseI is one of the key enzyme in the fatty acid elongation process. FabI is also inhibited *in vitro* by long-chain acyl-CoA (Bergler et al., 1996) and this mechanism may be relevant to regulate the *de novo* fatty acid synthesis when cells are grown in the presence of exogenous fatty acids, although the hypothesis has not yet been verified *in vitro* (Heat et al., 2001). The selective inhibition of the incorporation of labelled acetate into fatty acids especially in *de novo* long chain fatty acids by a chemical compound can be an indication of the FabI inhibition. Hence, the main aim of this experiment was to evaluate the influence of selected exogenous free fatty acids on the extracellular release of esterase by *Staphylococcus aureus* and on the incorporation of acetate into the *de novo* cellular fatty acids.

VI-2 Material and Methods

Chemical reagents and compounds were all purchased from Sigma-Aldrich (Milano Italy), while bacterial medium was from Oxoid (Basingstoke, UK).

VI-2-1 Minimal medium for *S. aureus*

A minimal medium was prepared for *S. aureus*, based on the paper of Robert et al. (1967). The composition and realization was as follow.

The Minimal Medium was based on a 800 ml salt solution containing glucose (10g) to which 100 ml of the amino acid solution and 100ml of the vitamins solution were added.

Preparation of salt solution for 800 ml of distillate water

a- KH_2PO_4……..0.8 g
b- Na_2HPO_4……2.64 g
c- NH_4Cl…….0.4 g
d- $MgSO_4.7H_2O$……..0.56 g
e- $FeSO_4.7H_2O$……0.008 g
f- NaCl……0.8 g
g- Glucose……10 g

At the end of the dissolution, the solution was made up to 800 ml and filter sterilized with a 0.2 μm disposable filter.

For the preparation of the Amino acid mix (1L), 250 mg of each of the following amino acids (glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteina, methionine, proline, arginine, histidine) were dissolved in most of the water, adding the next one only when the previous one had totally dissolved. At the end, the solution was made up to 1L and filter sterilized (0.2 μm).

Preparation of the vitamin mix (1L)

a- Thiamine HCl…….....0.33 mg
b- Nicotinic acid…….12 mg
c- Biotin……...0.03 mg
d- Riboflavin……0.04 mg

Vitamins were dissolved in most of the water, adding the next one only when the previous one has dissolved totally. At the end, the solution was made up to 1L and filter sterilized.
For the final minimal medium, 100 mL of amino acid solution and 100 mL of vitamin solution were dded to 800 mL of salt solution. The pH was corrected to 7 or 5 using HCl 5M and the various solutions filter sterilized before their use. The amino acid and vitamin solutions were stored at 2°C during the period of experiment.

VI-2-2 Bacterial growth

Staphylococcus aureus strain SR 231 stored at -80°C in BHI + 40% glycerol, was grown in BHI at 37°C for 24 h and successively incubated in minimal medium (pH 7) at 37°C for 24 h before being used for the experiment.

VI-2-3 Supernatant for extracellular esterase analysis

1 ml of *S. aureus* pre-inoculum, obtained as described previously, was inoculated in 9 ml of minimal medium pH 7 and incubated statically at 37°C until the cells were at the OD mid exponential phase (figure VI-2). 1ml of these cells were then inoculated in minimal medium at pH 7 and at pH 5, containing no free fatty acid (control samples) or 2 and 4 ppm of linolenic, lauric and capric acids dissolved in ethanol (96%). The cell free supernatants of the samples were obtained after 4, 7 and 24 hours of incubation at 37°C by separating the cells using a 0.2 µm disposable filter.

VI-2-4 Extracellular esterase

The extracellular concentration of esterase was assessed indirectly by measuring the quantity of paranitrophenol (PNP) obtained after hydrolysis of paranitrophenol acetate (PNP-Ac) after 1 hour of incubation at 30°C. The esterase experimental protocol was a slight modification of the assays of Higgins and Lapides (1947) and Krish (1966). A sample (1 ml) of cell free supernatant containing the enzyme was mixed with 0.9 ml of phosphate buffer (75 mM, pH 7.0) containing 10 mM $MgSO_4$. The reaction was started by the addition of 0.1 ml of 30 mM PNP-Ac in absolute ethanol at 30°C and was followed by recording the continuous changes in absorbance every 5 min for 1 hour at 405 nm in an UV-visible spettrophotometer 1601 (Shimadzu Italia S.r.l.) The quantity of PNP released was calculated using the difference of OD after 1 hour of reaction and the PNP standard curve regression equation (figure VI-1). The concentration of esterase was expressed as Unit per ml (U/ml) where 1 unit corresponded to 1 µM of PNP hydrolyzed by the esterase contained in 1 ml of supernatant after 1 hour at 30°C.

Figure VI-1: Paranitrophenol (PNP) optical density (405nm) linear regression equation at 30°C.

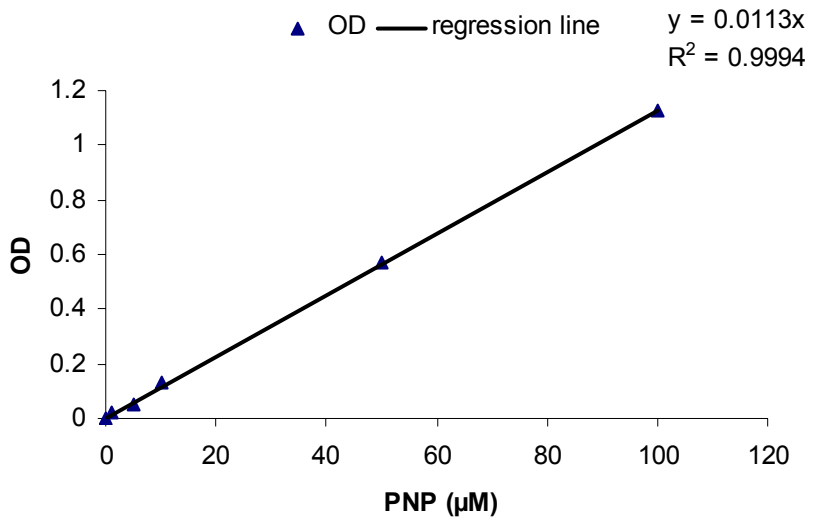
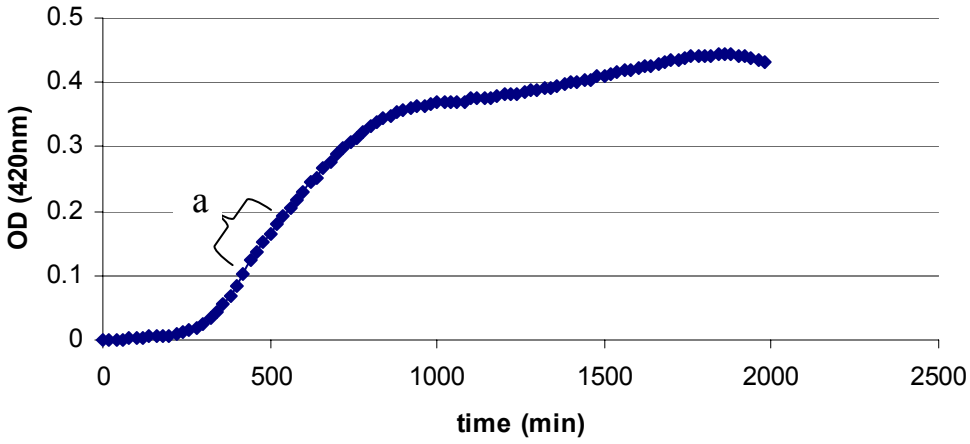


Figure VI-2: OD growth curve of *S. aureus* in minimal medium pH 7 at 37°C.



a- interval of cell harvesting

VI-2-5 *S. aureus* growth for de novo fatty acids assessment

Bottles of 49 ml of minimal medium at the desired pH containing 0, 2 and 4 ppm (adding a constant volume of fatty acid /ethanol solution of 0.5 ml) of the following free fatty acids, i.e. linolenic, lauric and capric acids, were added with 200 ppm of 1-^{13}C-2-d_3 sodium acetate as

indicated in the experimental plan in Table 1. 0.5 ml of *S. aureus* at the mid OD exponential phase was then inoculated (corresponding to a final cell load in the bottles of about 8 Log CFU/ml) in the different conditions as defined in table 1 and incubated statically at 37°C for 24 hours.

VI-2-6 Cellular fatty acid extraction and methylation

After incubation, the cells were harvested by centrifugation at 8000g for 15 min and the cells washed with two cycles of centrifugation at the same conditions using phosphate buffer (75 mM, pH 7.0). The cells were then weighed and a direct methylation of the cellular fatty acids was performed using the Microbial Identification System (MIS) produced by Microbial ID (MIDI, Newark, DE, USA). Briefly, a maximum of 50 mg of biomass harvested was transferred into a tube with a Teflon-lined cap using 1 ml of MIDI Reagent 1 (Table VI-2) and a disposable glass pipet. After shaking the tubes were placed in a boiling water bath for 5 min, shaken again and returned to the bath for 30 min. After cooling in a water bath 2 ml of MIDI Reagent 2 (Table VI-2) was added And the tube sealed, vigorously shaken and heated at 80 °C for 10 min. After cooling in a water bath, 1,5 ml of MIDI Reagent 3 (Table VI-2) was added and the tubes vigorously shaken. The bottom phases were removed with the a pipettor (0.5 ml) and discarded. Then 1 ml of the organic phase was transferred in another conical glass tube with Teflon cap and 3ml of MIDI Reagent 4 (Table VI-2) added and the tubes vigorously shaken. 490 µl of the top phases were transferred to chromatography vials and stored at -20°c until analysis.

VI-2-7 Gas chromatography analyses

The fatty acid methyl ester analysis was performed using the selected ions monitoring (SIM) mode and an Agilent Technology gas-chromatograph 6890N (Palo Alto, CA, US), equipped with an Agilent Network Mass Selective detector HP 5973 (Palo Alto, CA, US) and a capillary column (Supelcowax-10 30m x 0.32 mm x 0.25 µm). The injector and the detector were both held at 240°C and the temperature was programmed from 50°C (held for 1 min) to 220°C at a rate of 4°C/min and held at 220°C for 10 min. The carrier gas was helium with a rate of 1.2ml/min and a split 1:20. The selected ions used for non labelled fatty acids were m/z 55, m/z 87, m/z 69, m/z 74, m/z 83 and m/z 241, while for labelled fatty acids the target ions were m/z 57, m/z 89, m/z 43, m/z 76, m/z 85 and m/z 243. Identification and retention times were obtained by comparing those of the samples with those of the standards BAME and FAME mix from Sigma-Aldrich (Milan, Italy) analysed in SCAN mode and confirmed in SIM mode with the ions ratios. Calibration lines equations were obtained for ion 87 of each fatty acid by analyzing different concentrations of the FAME mix standard and single

branched chain fatty acids. Both non labelled and labelled fatty acids quantification was assessed using ion m/z 87 calibrations equation and expressed as μg per mg of harvested cells.

Table VI-1: Experimental plan for the assessment of labelled acetate in *de novo* fatty acids biosynthesis.

			Free fatty acids (ppm)		
Samples	acetate -1-^{13}C-2-d_3(ppm)	pH	C18:0	C12:0	C10:0
00-FApH7	0	7	0		
0-FApH7	200	7	0		
2-C18:3pH7	200	7	2		
4-C18:3pH7	200	7	4		
2-C12pH7	200	7		2	
4-C12pH7	200	7		4	
2-C10pH7	200	7			2
4-C10pH7	200	7			4
00-FApH5	0	5	0		
0-FApH5	200	5	0		
2-C18:3pH5	200	5	2		
4-C18:3pH5	200	5	4		
2-C12pH5	200	5		2	
4-C12pH5	200	5		4	
2-C10pH5	200	5			2
4-C10pH5	200	5			4

Table VI-2: Composition of the MIDI reagents.

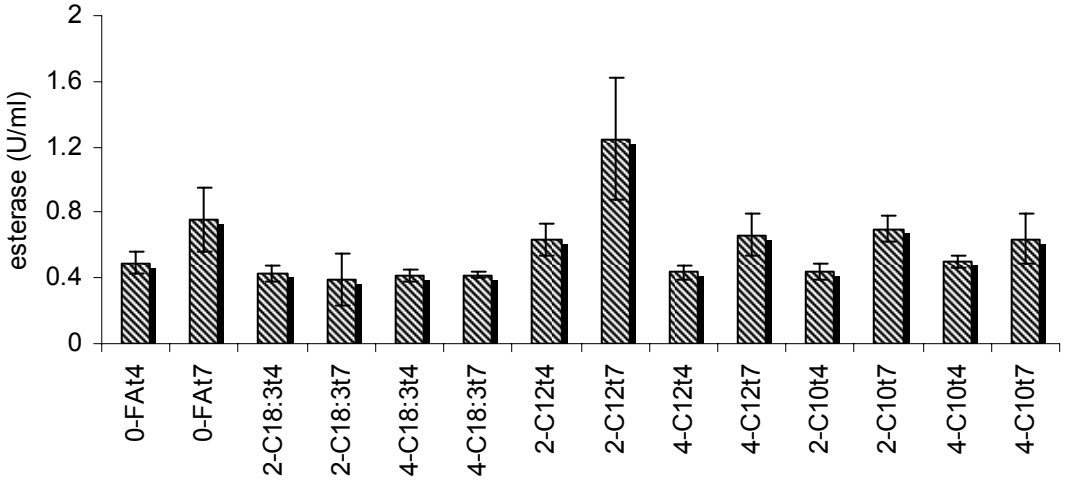
Reagent	Ingredient	Quantity
Reagent 1	NaOH	45g
	methanol	150 ml
	deionized water	150 ml
Reagent 2	6 N hydrochloric acid	325 ml
	methanol	275 ml
Reagent 3	hexane	200 ml
	methyl *tert*-butyl ether	200 ml
Reagent 4	sodium hydroxide	10.8 g
	deionized water	900 ml

VI-3 Results

VI-3-1 Influence of free fatty acids on the release of extracellular esterase

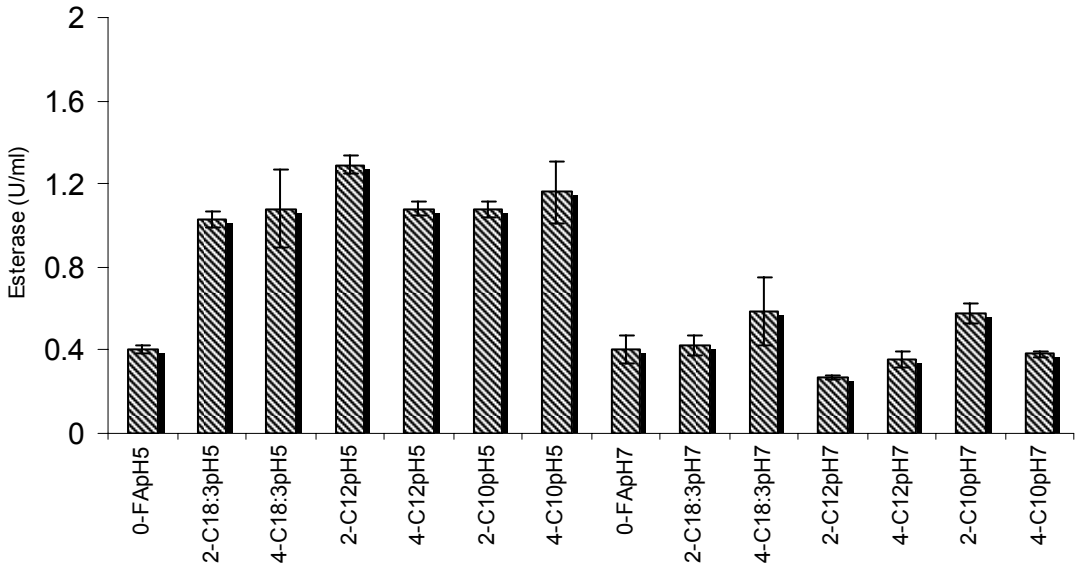
In figure VI-3 the esterase released in the presence of 0, 2 and 4 ppm of α-linolenic, lauric and capric acids at pH 7 after 4 and 7 hours at 37°C are reported. Except in the case of *S. aureus* in the presence of α-linolenic acid, the extracellular concentration of esterase increased with the incubation time. Under this conditions, no statistical differences were observed between the esterase released in the control samples with respect to the other samples in the presence of the free fatty acids.

Figure VI-3: Extracellular esterase concentrations after 4 and 7 hours of incubation at 37°C (pH 7) of *S. aureus* in the presence of 2 and 4 ppm of free fatty acids.



When the incubation time was prolonged to 24 hours (figure VI-4), no difference in the esterase released at pH 7 at the fatty acid concentrations tested was observed if compared with the control. At pH 5 after 24 hours of incubation, the units of esterase per ml were higher than those at pH 7 in the presence of free fatty acids, while the same concentration of the enzyme was observed at the two pH values in the absence of FFAs. Increasing the fatty acid concentration from 2 to 4 ppm had little influence on the extracellular concentration of esterase.

Figure VI-4: Extracellular esterase concentration after 24 hours of incubation at 37°C of *S. aureus* in the presence of 2 and 4 ppm of free fatty acids at pH 7 and pH 5.



VI-3-1 Influence of free fatty acids on the inclusion of labelled acetate in the *de novo* fatty acid biosynthesis of *S. aureus*

S. aureus was grown in the presence of selected free fatty acids with only glucose and labelled acetate as carbohydrate sources. The cell fatty acids were analysed as previously described in order to quantify the fatty acids containing one group of ^{13}CHd as the result of the inclusion of $^{13}Cd_3COO^- N_a^+$ in the *de novo* fatty acid biosynthesis. The fatty acid percentage composition of *S. aureus* at pH 7 and pH 5 were analysed from the samples 00-FApH7 and 00-FApH5, respectively (Table VI-3). When grown at pH 7, *S. aureus* cells were composed principally of long chain saturated fatty acids (more than 65%) and *anteiso* and *iso* fatty acids. At pH 5, the percentage composition of *anteiso* and *iso* fatty acids was reduced while that of long chain saturated fatty acids was increased with respect to the cell composition of bacteria grown at pH 7. Moreover the ratio branched/unbranched fatty acid in *S. aureus* cell lipids was 0.39 and 0.22 at pH 7 and pH 5, respectively.

Table VI-3: Percentage composition of *S. aureus* cell fatty acids grown at pH 7 and pH 5.

Fatty acids (percentage of total fatty acids)	pH7	pH5
C13:0	trace	trace
C14:0	4.25±0.7	7.84±1.72
iC15:0	13.91±0.18	7.92±2.25
aC15:0	11.37±2.89	9.52±0.5
C15:0	2.07±0.38	2.54±0.08
iC16:0	1.52±0.02	0.69±0.17
C16:0	30.28±3.88	38.27±4.99
C16:1	trace	trace
iC17:0	1.83±0.26	0.32±0.11
aC17:0	0.79±0.19	0.38±0.14
C17:0	2.15±0.39	1.71±0.09
C18:0	26.42±0.3	30.22±0.17
cis C18:1	0.73±0.18	0.93±0.02
C19:0	3.07±0.54	0.91±0.05
C20:0	7.77±0.41	4.76±0.9

In the presence of labelled acetate, *de novo* fatty acids containing the labelled components synthesized by *S. aureus* after 24 hours of incubation at 37°C were quantified using the ion m/z 89. ions m/z 87 and m/z 89 chromatograms of cells fatty acids extract obtained after growing *S. aureus* in the absence of labelled (00-FApH7) and in the presence of labelled acetate (0-FApH7) are reported as examples in appendix VI-2 and appendix VI-3 respectively. In tables VI-4 and VI-5 the cellular labelled fatty acids after incubation at pH 7 and pH 5 are reported, respectively. At pH 7 incorporation of acetate was not observed only in the cells unsaturated fatty acids, while the level of labelled compound biosynthesis differed with the type of fatty acids added in the medium. In particular, labelled C14:0 was detected more when the cells were incubated in the presence of lauric and capric acids than when in presence of α-linolenic and in the control.

Table VI-4 : *S. aureus de novo* fatty acids after 24 hours of incubation at pH 7 in the presence of selected free fatty acids.

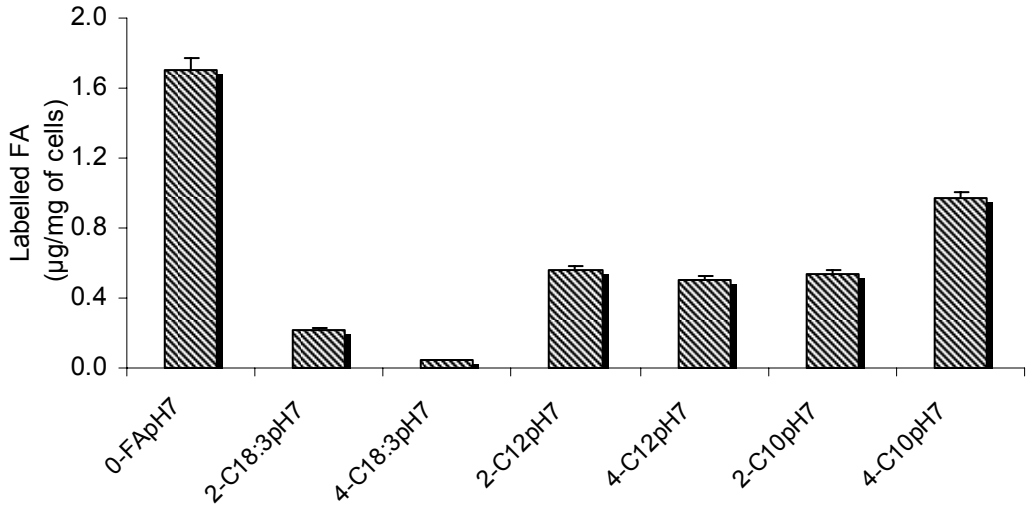
Fatty acid (µg/mg of cells)	**0-FApH7**	**2-C18:3pH7**	**4-C18:3pH7**	**2-C12pH7**	**4-C12pH7**	**2-C10pH7**	**4-C10pH7**
C14:0	0.019±0.001	0.013±0.004	traces	0.243±0.012	0.222±0.007	0.192±0.003	0.392±0.005
*i*C15:0	0.695±0.012	0.078±0.0023	0.039±0.0031	0.056±0.002	0.059±0.0031	0.047±0.0025	0.093±0.0016
*a*C15:0	0.227±0.022	0.054±0.0017	0.008±0.0013	0.037±0.003	0.031±0.002	0.064±0.0017	0.079±0.0022
C15:0	0.020±0.01		traces	traces		0.003±0	0.001±0
*i*C16:0	0.069±0.005	0.003±0.001					
C16:0	0.044±0.002	traces	traces	0.113±0.006	0.101±0.007	0.109±0.005	0.213±0.004
C16:1							
*i*C17:0	0.056±0.0021						
*a*C17:0	traces	traces	traces	traces	traces	traces	traces
C17:0	0.051±0.002				0.0003±0	traces	0.0003±0
C18:0	0.258±0.0031	0.005±0.0014	traces	0.067±0.002	0.058±0.0013	0.092±0.003	0.150±0.011
cis C18:1							
C19:0	0.038±0.0013						0.006±0
C20:0	0.229±0.0011	0.063±0.0012	traces	0.042±0.001	0.037±0.0015	0.031±0.004	0.042±0.001
Total	**1.707±0.062**	**0.217±0.012**	**0.047±0.004**	**0.558±0.03**	**0.508±0.022**	**0.539±0.019**	**0.976±0.025**

Table VI-5: *S. aureus de novo* fatty acids after 24 hours of incubation at pH 5 in the presence of selected free fatty acids.

Fatty acid (µg/mg of cells)	**0-FApH5**	**2-C18:3pH5**	**4-C18:3pH5**	**2-C12pH5**	**4-C12pH5**	**2-C10pH5**	**4-C10pH5**
C14:0	0.007±0	traces	traces	0.104±0.006	0.014±0.0018	0.368±0.012	0.232±0.011
*i*C15:0	0.009±0	traces	traces	0.041±0.002	traces	0.104±0.017	0.063±0.0024
*a*C15:0	0.168±0.005	0.002±0	traces	0.092±0.0015	0.005±0	0.145±0.021	0.057±0.005
C15:0		0.002±0			traces	0.002±0.0004	0.002±0
*i*C16:0		0.002±0	0.002±0.0003	0.003±0.001	traces	traces	0.0005±0
C16:0	0.090±0.003	0.011±0.0022	0.016±0.003	0.096±0.0023	0.019±0.003	0.170±0.021	0.100±0.031
C16:1							
*i*C17:0							
*a*C17:0	traces	0.003±0	traces	traces	traces	traces	traces
C17:0					traces	0.004±0	0.002±0
C18:0	0.191±0.013	traces	0.002±0.001	0.090±0.008	0.011±0.002	0.177±0.006	0.086±0.009
cis C18:1							
C19:0							
C20:0						0.067±0.004	0.039±0.006
Total	**0.465±0.02**	**0.019±0.002**	**0.020±0.003**	**0.426±0.021**	**0.050±0.006**	**1.038±0.081**	**0.581±0.064**

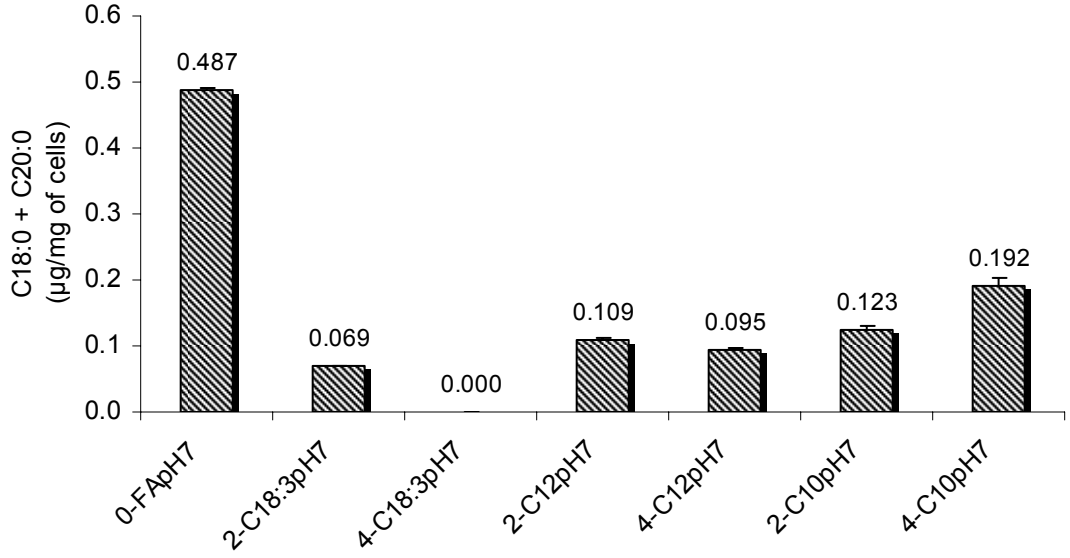
Regarding the *anteiso* and *iso* branched fatty acids, they were observed prevalently in the control samples, indicating a reduction in their biosynthesis when *S. aureus* was grown at pH 7 in the presence of fatty acids. In particular, *i*C17:0 was not synthesized in the presence of exogenous fatty acids, while *a*C17:0 was detected only in traces in all the samples analysed. In figure VI-5 the total fatty acids *de novo* synthesized in the different conditions tested are reported. The fatty acids biosynthesis was inhibited by the presence of exogenous fatty acids with respect to the control. Only in the presence of α-linolenic acid a reduction of the *de novo* synthesis was observed when the concentration of the exogenous fatty acid increased.

Figure VI-5: Total fatty acids synthesized in the presence of exogenous fatty acids at pH 7.



Considering only C18:0 and C20:0, their *de novo* synthesis was totally inhibited when 4 ppm of α-linolenic acid were added to the minimal medium of *Staphylococcus aureus* and grown at 37°C for 24 hours (figure VI-6). In the presence of the other exogenous free fatty acids, there was an inhibition of the synthesis with respect to the control, but it was constant when increasing the concentration of lauric acid. On the contrary C18:0 and C20:0 synthesis was increased in the presence of the highest capric acid concentration in the medium.

Figure VI-6: Sum of C18:0 and C20:0 synthesized in the presence of exogenous fatty acids at pH 7.



At pH 5, the *de novo* fatty acid synthesis was in general reduced with respect to that at pH 7. Only C16:0 and C18:0 were detected in all the experimental conditions while the other fatty acids were synthesized particularly in the control samples and when capric acid was added to the medium (figure VI-5). Regarding the total fatty acids synthesized when *S. aureus* was exposed to free fatty acids at pH 5 (figure VI-7; table VI-5), it can be observed that α-linolenic acid totally inhibited the *de novo* synthesis of all the FAs. In the presence of 2 ppm of lauric acid, the fatty acid biosynthesis showed no difference with that of cells in the control samples, while the same concentration of capric acid activated the *de novo* fatty acid synthesis. In general, increasing concentrations of the exogenous fatty acids resulted in lower biosynthesis rates. Long chain saturated fatty acid (C18:0 synthesis was totally inhibited in the presence of α-linolenic acid and it was also inhibited as the concentration of the exogenous lauric acid added to the medium increased. On the contrary to what observed at pH 7, the synthesis of C18:0 plus C20:0 was reduced with increasing concentrations of capric acid at pH 5 (figure VI-8).

Figure VI-7: Total fatty acids synthesized in the presence of exogenous fatty acids at pH 5.

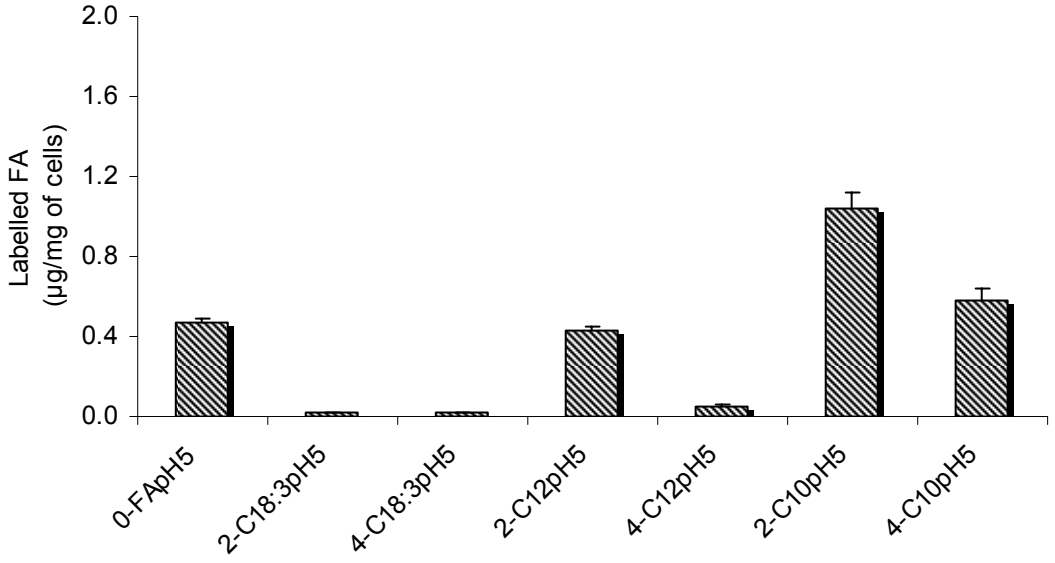
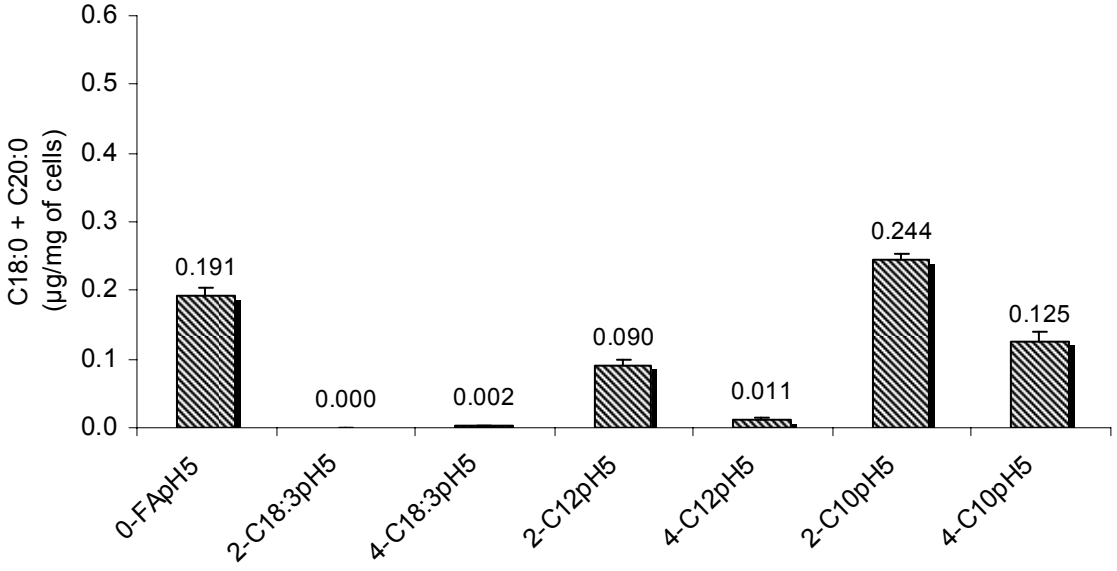


Figure VI-8: Sum of C18:0 and C20:0 synthesized in the presence of exogenous fatty acids at pH 5.



VI-4 Discussion

Esterases differs from lipases in that they hydrolyse small ester containing molecules partially soluble in water like short-chain acylglycerides, whereas lipases have an optimal activity towards long chain triacylglycerides not soluble in such aqueous environment (Jaeger et al., 1999). Although the physiological function of many microbial esterase remains not clear, some of these enzymes are known to be involved in metabolic pathways that provide access to carbon sources. Such enzymes include the acetyl and cynnamoyl esterases that are involved in the degradation of hemicellulose (Dahymphe et al., 1996). In some plant pathogenic bacterial and fungal strains, these cell wall degrading esterase activities are believed to be pathogenic factors (Mc Queen et al., 1997). Detoxification of biocides may be another important role of esterase in microorganism (Blackman et al., 1995). Among other properties, esterases differs in substrate specificity and cellular location. In fact according to Casaburi et al. (2006) the different esterases activities between strains of different staphylococci species is probably due to the esterase location. Membrane disruption can lead to the release in the (extracellular) medium of both cell envelope associated and intracellular enzymes. At inhibitory concentrations, low acids are known to act by causing disorder in the bacterial membrane. But at concentrations under the minimum inhibitory concentration, their interaction with the cell membrane is not well documented. In the minimal medium used in this experiment, no component was able to induce the release of esterase in order to increase the carbon source by hydrolysis. The concentration of esterase found in control samples can then be assumed as the normal release observed during bacterial growth. At pH 7, no difference was observed in the extracellular release of esterase within samples collected after 4 hours of incubation and also was within those analysed after 7 hours. This indicates that the free fatty acids added in the growth medium did not cause any membrane disorder able to influence the release of supplementary esterase even after 24 hours of incubation. The fatty acids concentration tested at pH 7 are under their minimal inhibitory concentration (MIC) and had a very low antimicrobial activity; hence they acted as bacteriostatic compounds than as bactericidal ones. Growing the cells at pH 5 in the presence of exogenous fatty acids increased the extracellular release of esterase with respect to what observed at pH 7. In acid conditions, the undissociated form of the fatty acids prevails over the dissociated form and, creating a more favourable condition for their penetration into the cell membrane. This increased cell membrane disorder due to the exogenous fatty acid presence enhances the release of cell envelope associated esterases and probably also of intracellular enzymes. The cells response to this condition

is correlated to the cell membrane adaptation in order to increase the barrier property and reduce communication between intracellular and extracellular compartments.

One of the most important adaptation mechanism of bacteria in response to the changing environment is the modification of cell fatty acid composition (Rosas et al., 1980; Diefenbach et al., 1992; Donato et al., 2000). Although fatty acid biosynthesis occurs rapidly, it may take some time for the newly formed fatty acids to be incorporated into the membrane if the bacterium is not actively growing (Russel, 1994). In the presence of antimicrobials, the growth delay affects also the fatty acid biosynthesis and a long period of incubation is necessary to obtain a significant *de novo* synthesis. *Staphylococcus aureus* grown in minimal medium in static aerobic condition showed a predominance of straight and branched chain saturated fatty acids both at pH 7 and pH 5. The high percentage of straight saturated fatty acid with respect to the unsaturated and branched fatty acids conferred a more stable membrane with low fluidity compatible with the high temperature of incubation. In fact bacterial membranes with numerous saturated chains melt at higher temperatures than those composed primarily of unsaturated chains (Quinn, 1981). In the *S. aureus* cell fatty acids analysed, the fluidity is assured by the presence of branched chain fatty acids. From the percentage composition of the fatty acids, according to what observed by White and Frerman (1968), the growth of the cells in static conditions of our experiment can be considered more an anaerobic-like than an aerobic one . The low proportion of *anteiso* C15:0 with respect to what observed in literature can be correlated to the absence of isoleucine in the minimal medium. In fact this amino acid is the main primer source for the biosynthesis of *anteiso* C15:0 and *anteiso* C17:0 (Kaneda, 1991; Vlaeminck et al., 2006) The reduction of the ratio branched to unbranched fatty acids when passing from pH 7 to pH 5 is related to the ability to modulate the membrane fatty acid composition by increasing the straight chains and hence lowering the membrane fluidity and penetrability. Straight chain fatty acids can pack tightly side to side with respect to branched chain fatty acids by optimizing their van der Waals interaction (Freedman, 1981; Hazel and Williams, 1990).

The biosynthesis of *de novo* fatty acids is hence also a cell adaptation method of growth. The use of labelled acetate and other compounds related to the fatty acid biosynthesis to monitor the fatty acid biosynthesis pathway is a diffused practice (Heath et al., 1998; Payne et al., 2002). The single ion monitoring mode (SIM) related to a GC-Mass offers the possibility of tracing very low quantities of newly synthesized fatty acids containing the labelled group of the acetate used. Acetate is incorporated in the fatty acid through its transformation into acety-CoA and the condensation with malonyl-ACP by FabH to form β-ketobutyryl-ACP which is carried out in the biosynthesis

pathway. At pH 7 and pH 5, no *de novo* unsaturated fatty acids were synthesized, as also observed by Heath et al., (2001) according to whom Gram-positive bacteria do not make unsaturated fatty acids by *de novo* synthesis. The high biosynthesis of *de novo* C14:0 when *S. aureus* was grown in the presence of C12:0 and C10:0 both at pH 7 and at pH 5 can be interpreted as an indication of direct utilization of these fatty acids and their elongation through the addition of one or two malonyl-CoA respectively. The inhibition of this process of elongation in the presence of C18:3 with respect to the control and in the presence of the other fatty acids denote a reduction of the elongation process. Most Gram-positive bacteria contain *iso* and *anteiso* methylbranched-chain fatty acids instead of unsaturated straight-chain fatty acids and have FabH that selects for the branched-chain primers over acetyl-CoA (Han et al., 1998; Choi et al., 2000). It can be suggested that the inhibition of labelled acetate incorporation in branched chain fatty acids when *S. aureus* was grown in the presence of α-linolenic, lauric and capric acids is related to the FabH inhibition. In fact in the minimal medium the only primer source absent was isoleucine, correlated with *a*C15:0 and *a*C17:0 biosynthesis (Vlaeminck et al., 2006). Among the enzymes involved in the fatty acids biosynthesis, FabI is the one responsible for the elongation completion. Long chain fatty acids for their need of elongation during their synthesis, are more probably those which are more correlated to the activity of FabI. In this perspective, the incorporation of labelled acetate in the *de novo* synthesis of C18:0 and C20:0 can give information about the rate of FabI activity. In our experiment, the C18:3 considerably inhibited with a dose dependence-effect the *de novo* synthesis of these two fatty acids; on the contrary of C12:0 and C10:0 indicating a possible action on the FabI as also observed by Zheng et al., (2005). The different mode of action of the different exogenous free fatty acids is also observed on the overall cell membrane *de novo* fatty acids. A lowering the pH of the medium constrains the cell to adjust the membrane fatty acid composition with the increase of saturated fatty acids. However, microorganisms may survive in conditions of low pH, and although growth may have stopped, the cells may still be metabolically active. The energy requirements of a microorganism in a low pH environment are greater than the energy required at optimal pH values (Beales, 2004). This is because an energy-requiring proton pump is in use, with protons being pumped out of the cell. In high pH environments, protons may be pumped into the cell. If the pH is not balanced, the cell is unable to synthesize normal cellular components and is unable to divide and grow (Booth and Kroll 1989; Brown and Booth 1991). This is consistent with the low *de novo* synthesis observed at pH 5 with respect to pH 7 except when *S. aureus* was grown in the presence of capric acid. In our experiment, at the concentration tested, capric acid seem to stimulate the cell fatty

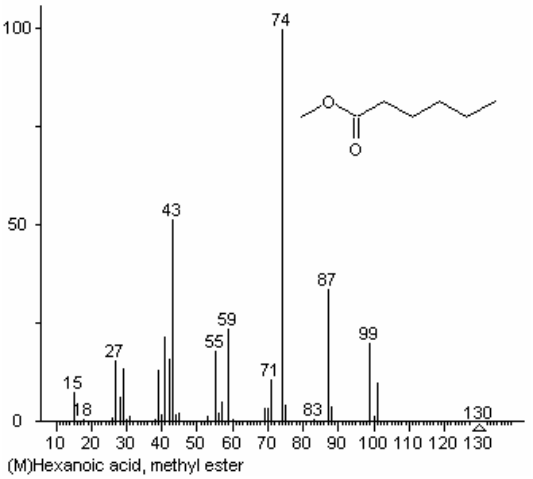
acid *de novo* synthesis with respect to the other free fatty acids tested and the control sample without any exogenous fatty acid.

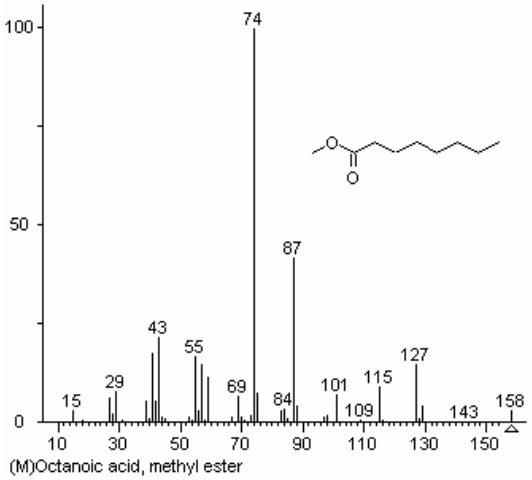
VI-5 Conclusion

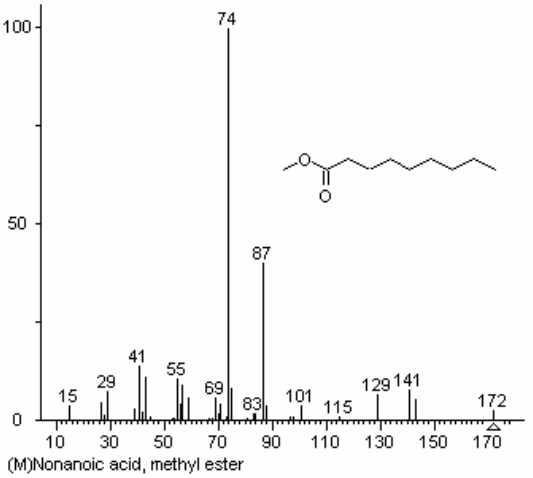
Fatty acid *de novo* synthesis is an important phase in the bacterial adaptation and growth. The presence of exogenous free fatty acids can enhance the adaptation difficulty by causing cell membrane disruption and possible release of cell envelope esterase in the extracellular medium. Non inhibitory concentrations of α-linolenic, lauric and capric acids induced an extra release of esterases only at low pH when the concentration of their undissociated form is higher. Although at pH 7 no evident membrane degradation (assed through the release of cell envelope esterases) was observed, the influence of exogenous free fatty acid was consistent on the *de novo* cell fatty acid biosynthesis. New evidences for the inhibition by α-linolenic acid of the fatty acid biosynthesis elongation completion enzyme FabI were also obtained. These results contribute to enlighten the complex mechanism of inhibition of free fatty acids and suggest that medium chain saturated fatty acids other than acting at the cell membrane level, may potentially interact with the cell fatty acids biosynthesis.

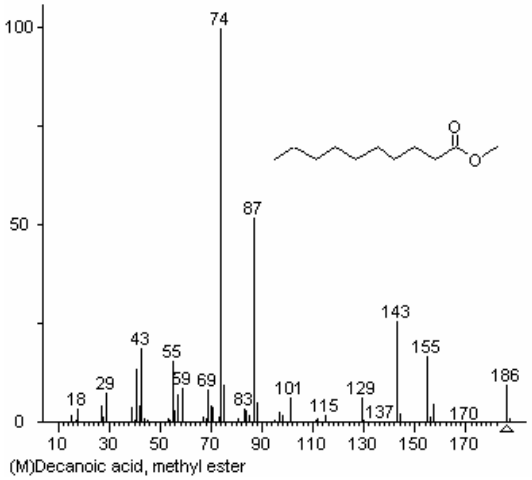
VI-6 Appendix

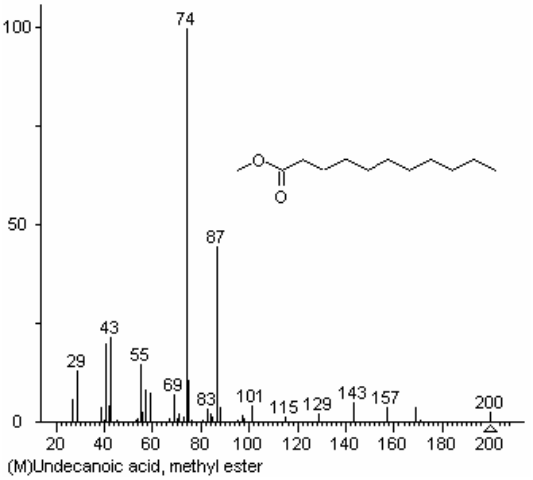
Appendix VI-1 Mass fragmentation of selected fatty acids methyl esters

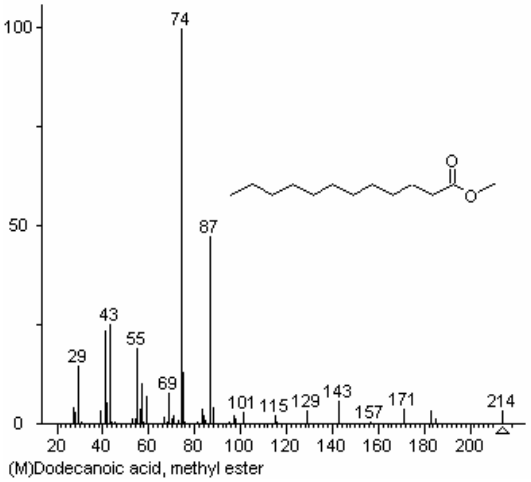


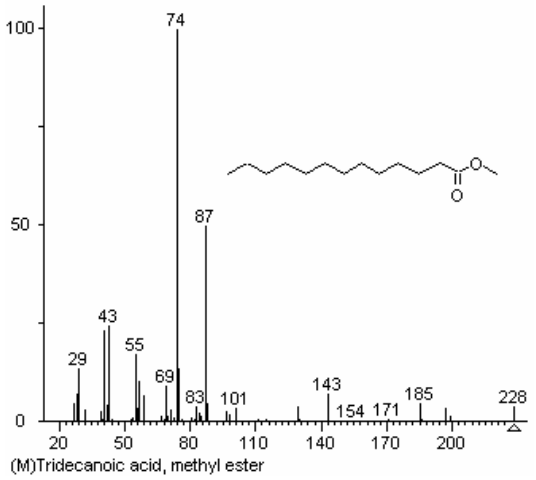




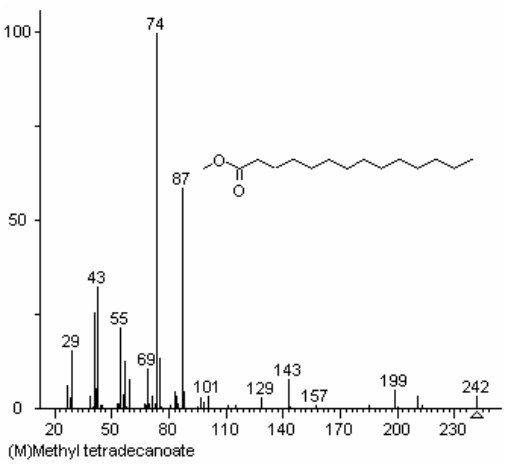




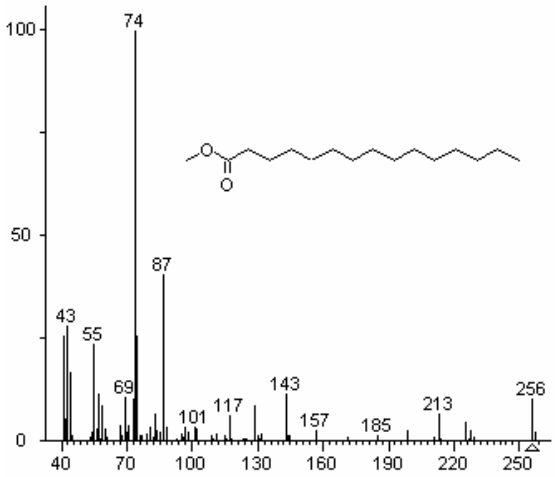




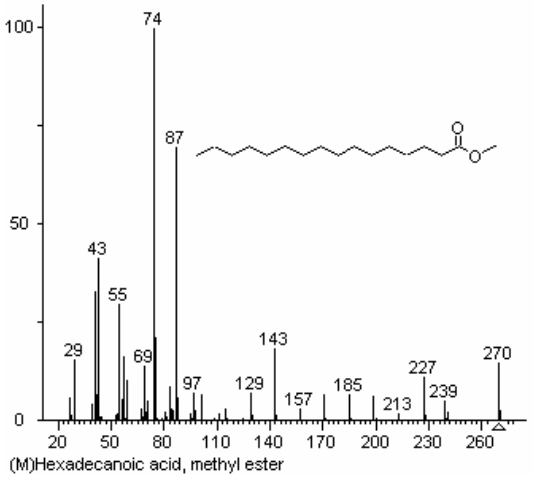
(M)Tridecanoic acid, methyl ester



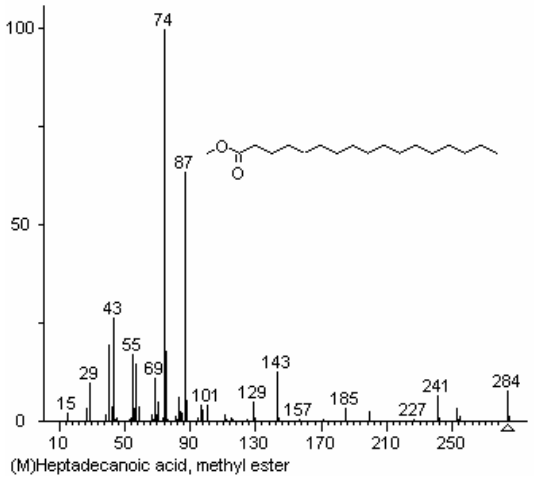
(M)Methyl tetradecanoate



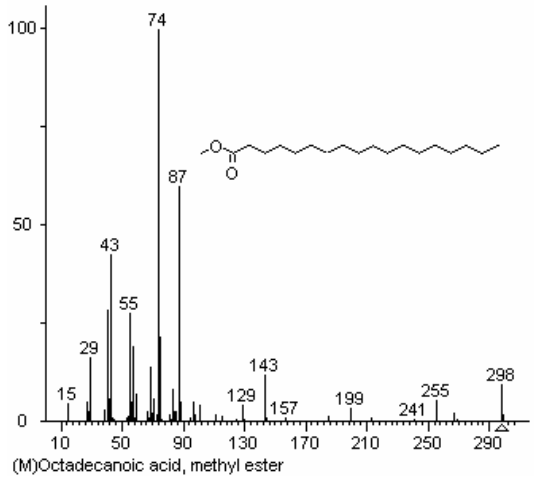
(M)Pentadecanoic acid, methyl ester



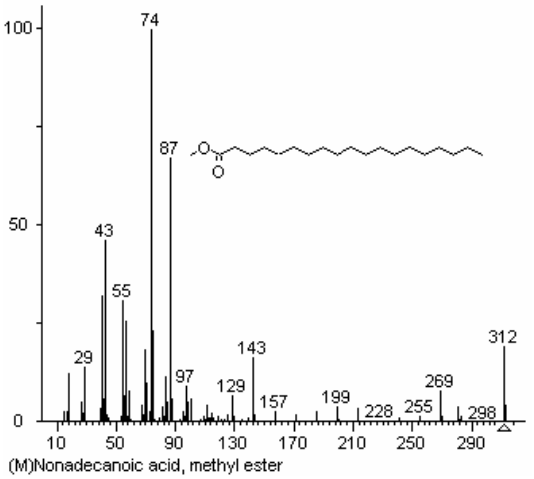
(M)Hexadecanoic acid, methyl ester



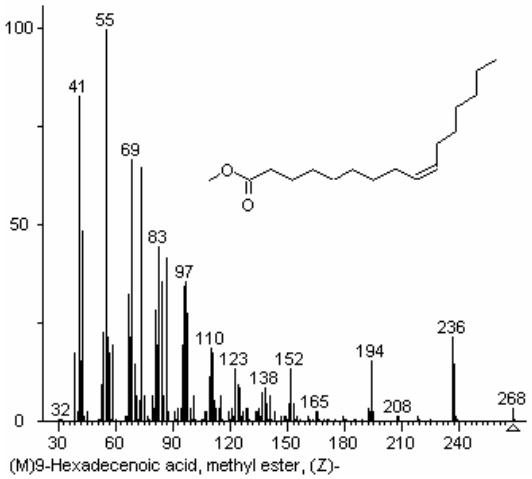
(M)Heptadecanoic acid, methyl ester



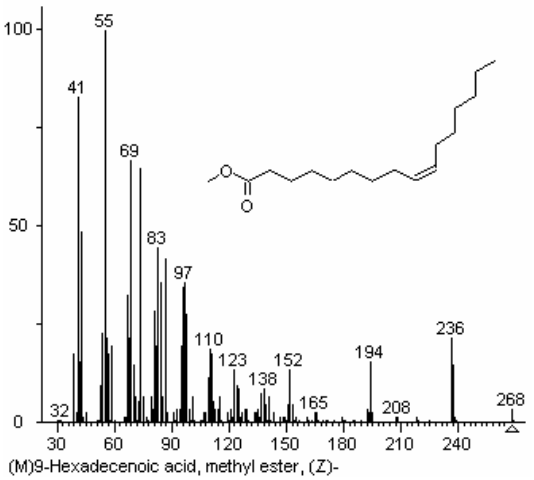
(M)Octadecanoic acid, methyl ester



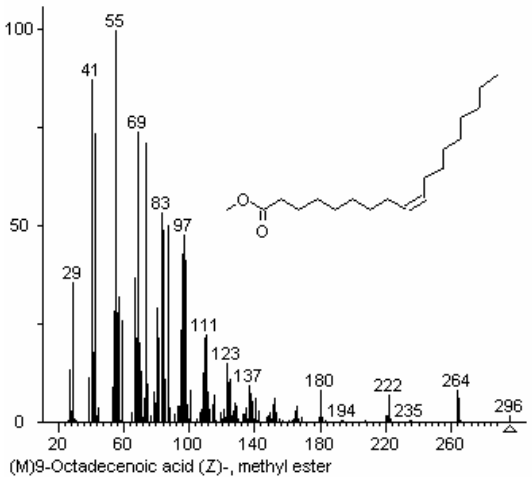
(M)Nonadecanoic acid, methyl ester



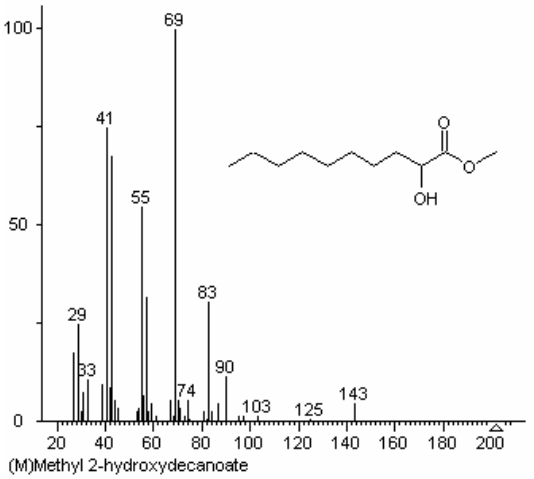
(M)9-Hexadecenoic acid, methyl ester, (Z)-



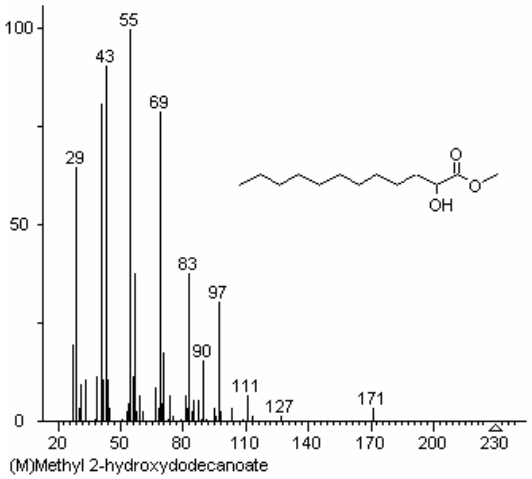
(M)9-Hexadecenoic acid, methyl ester, (Z)-



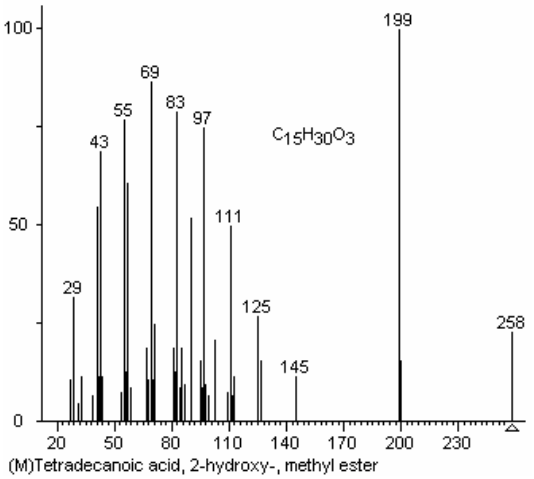
(M)9-Octadecenoic acid (Z)-, methyl ester

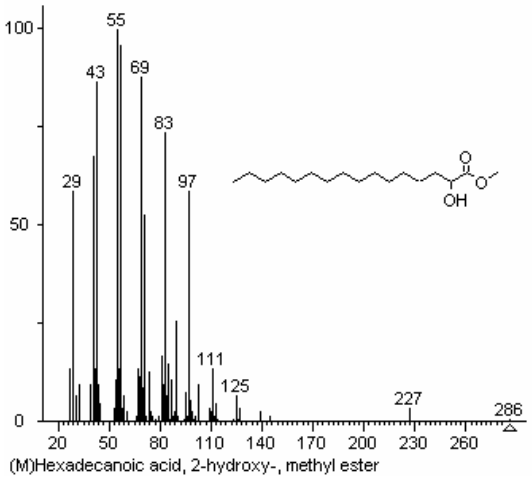


(M)Methyl 2-hydroxydecanoate

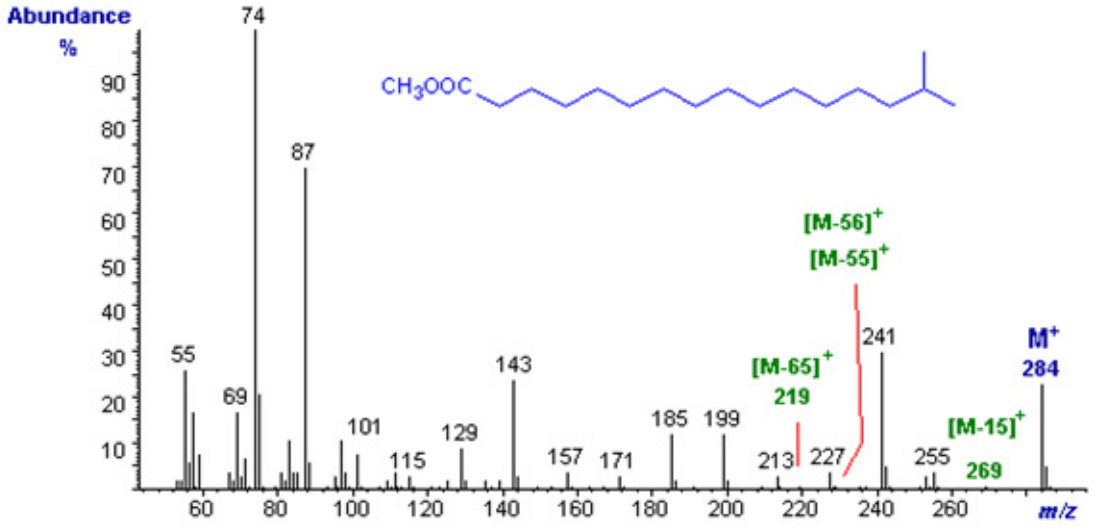


(M)Methyl 2-hydroxydodecanoate

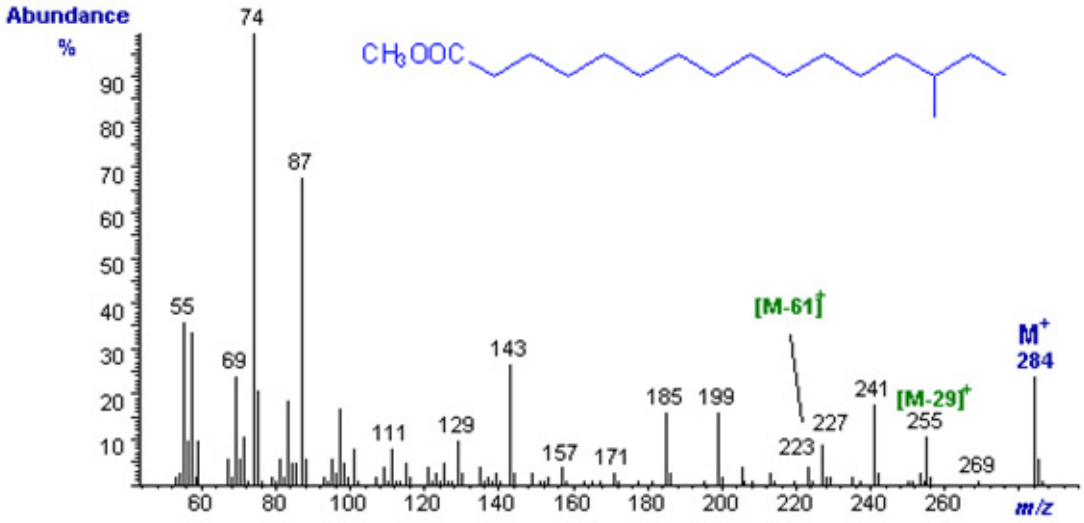




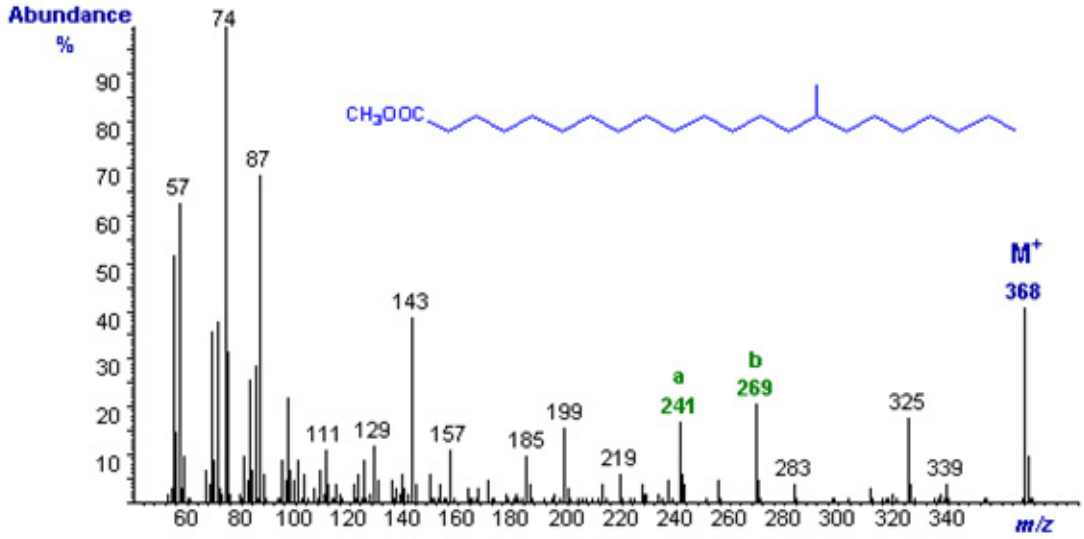
methyl *iso*-methylhexadecanoate (or 15-methylhexadecanoate)



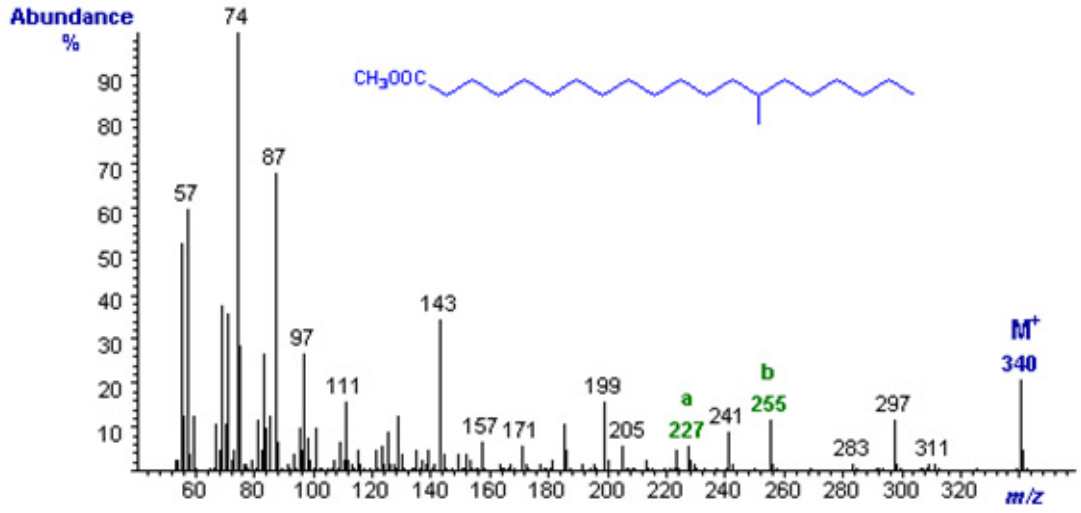
methyl *anteiso*-methyl-hexadecanoate (or 14-methyl-hexadecanoate)



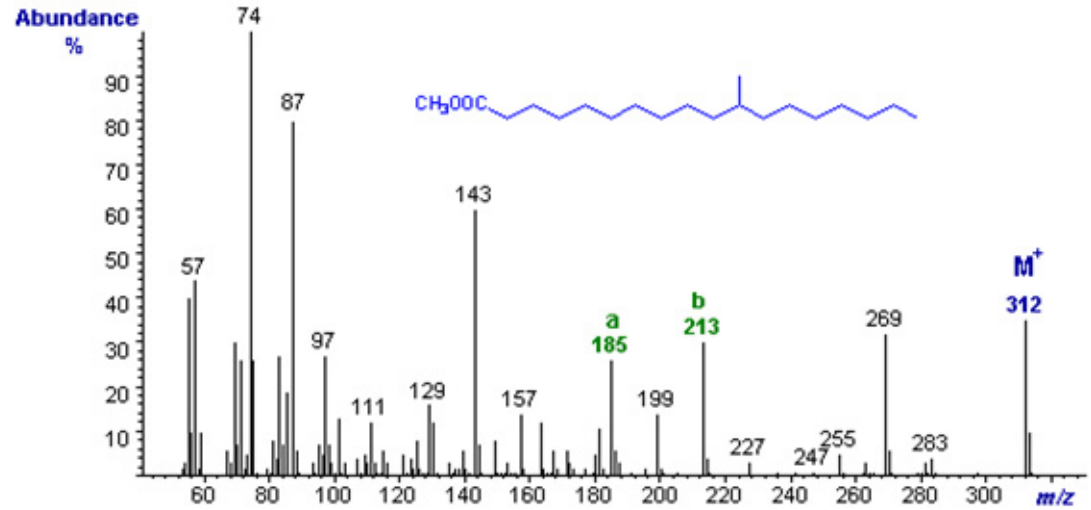
methyl 15-methyl-docosanoate

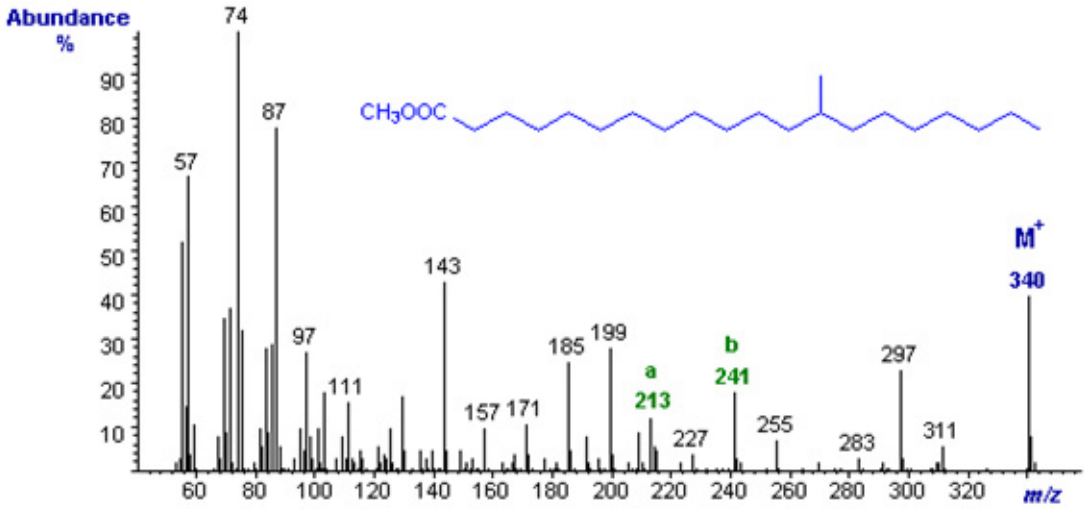


methyl 14-methyl-eicosanoate

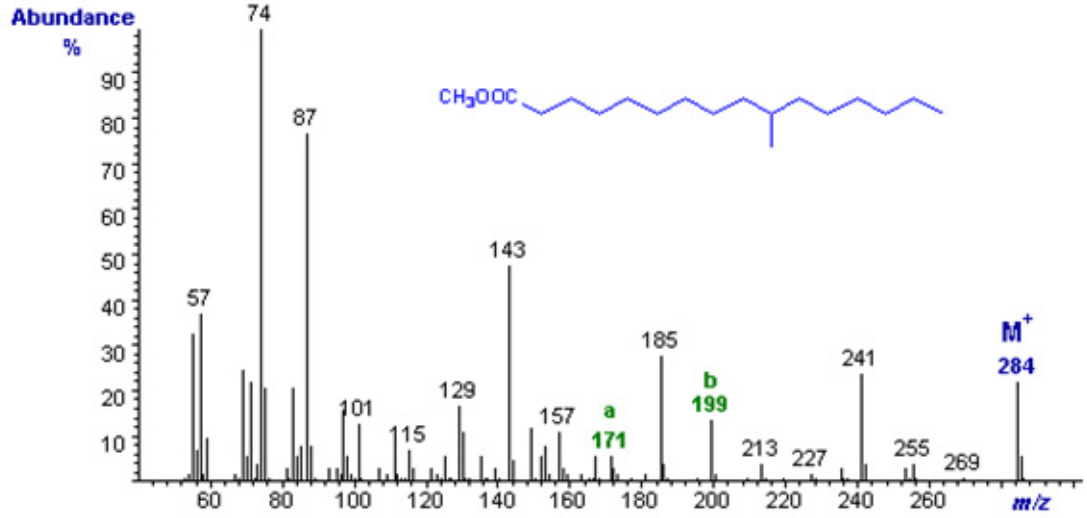


methyl 13-methyl-eicosanoate
methyl 11-methyl-octadecanoate

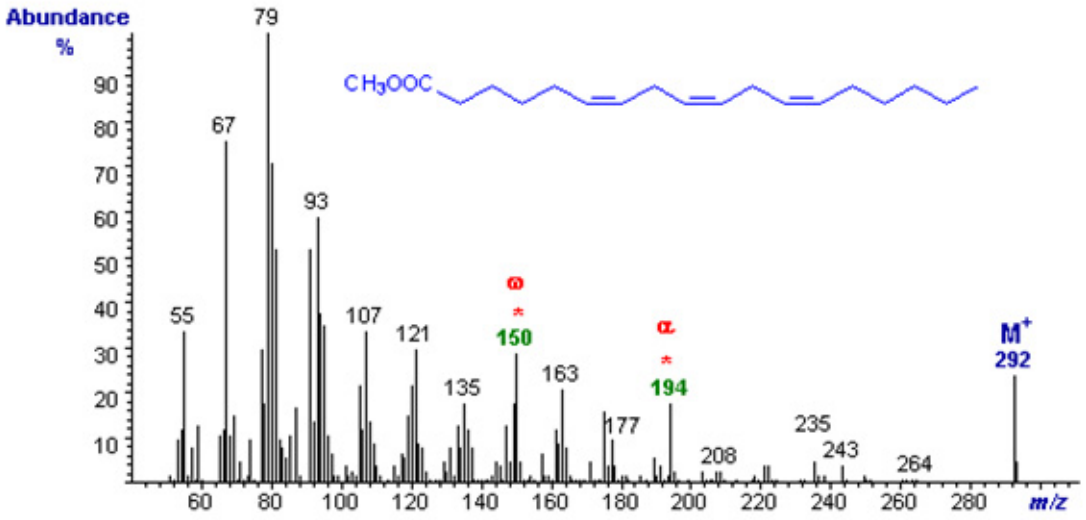




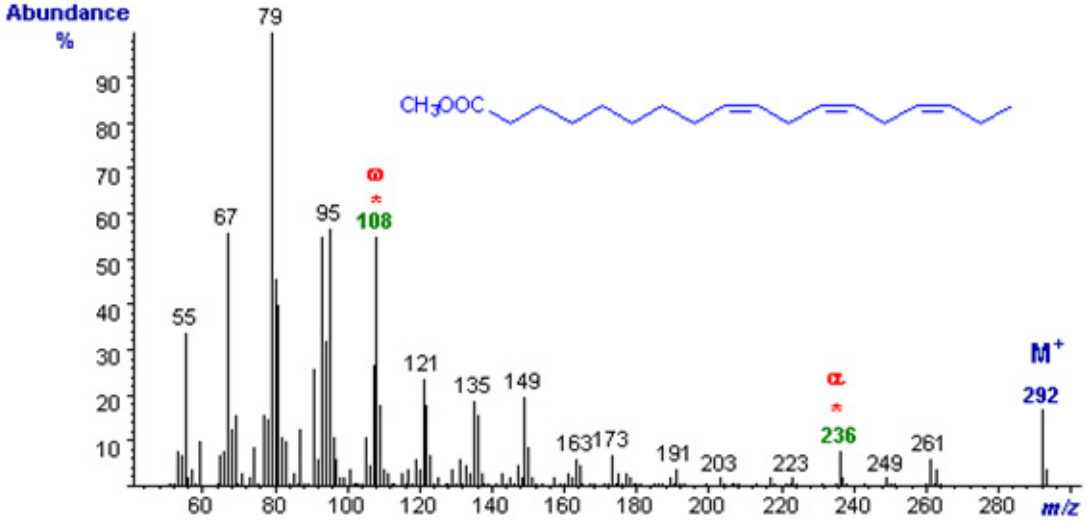
methyl 10-methyl-hexadecanoate



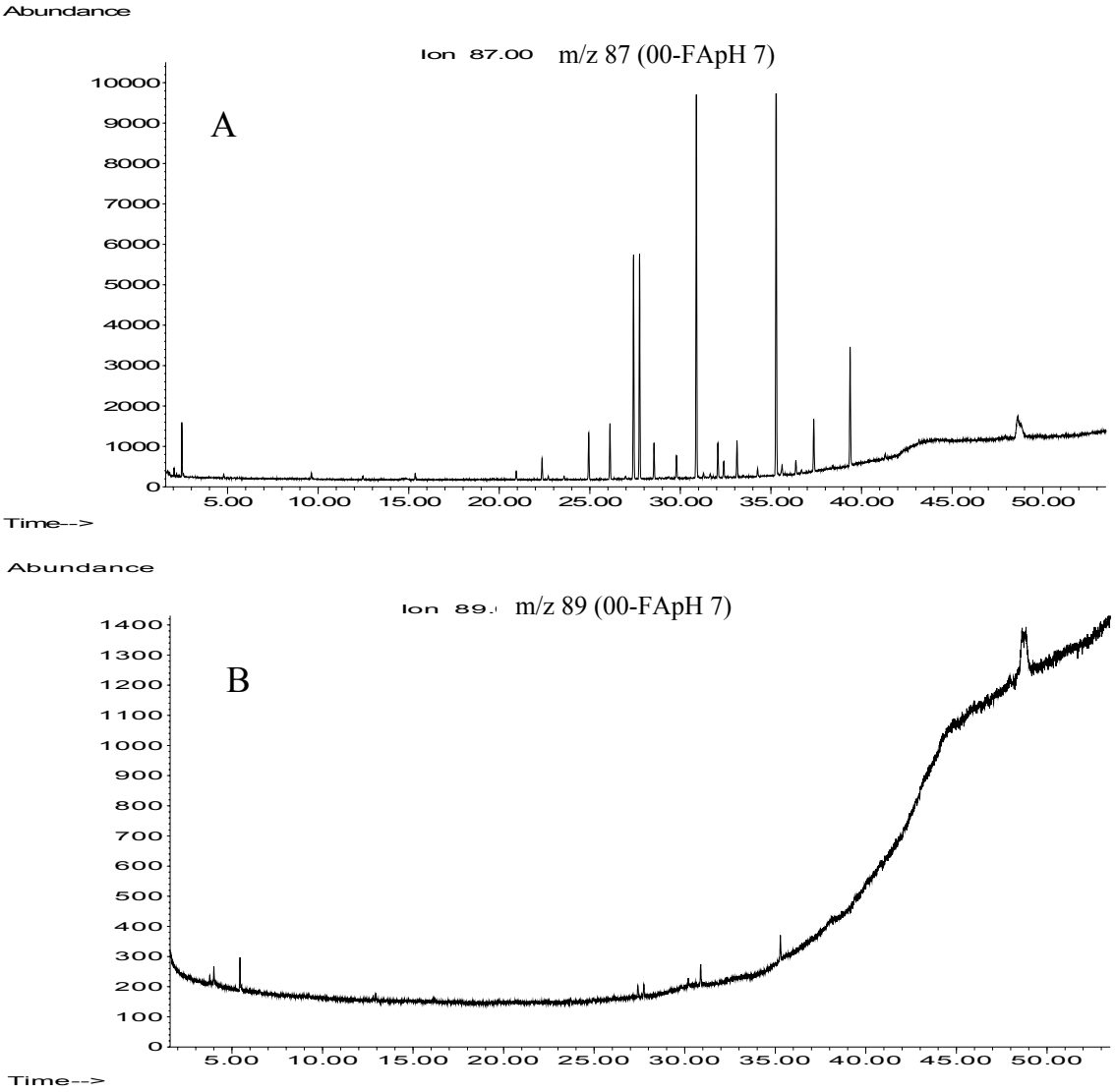
methyl 6,9,12-octadecatrienoate



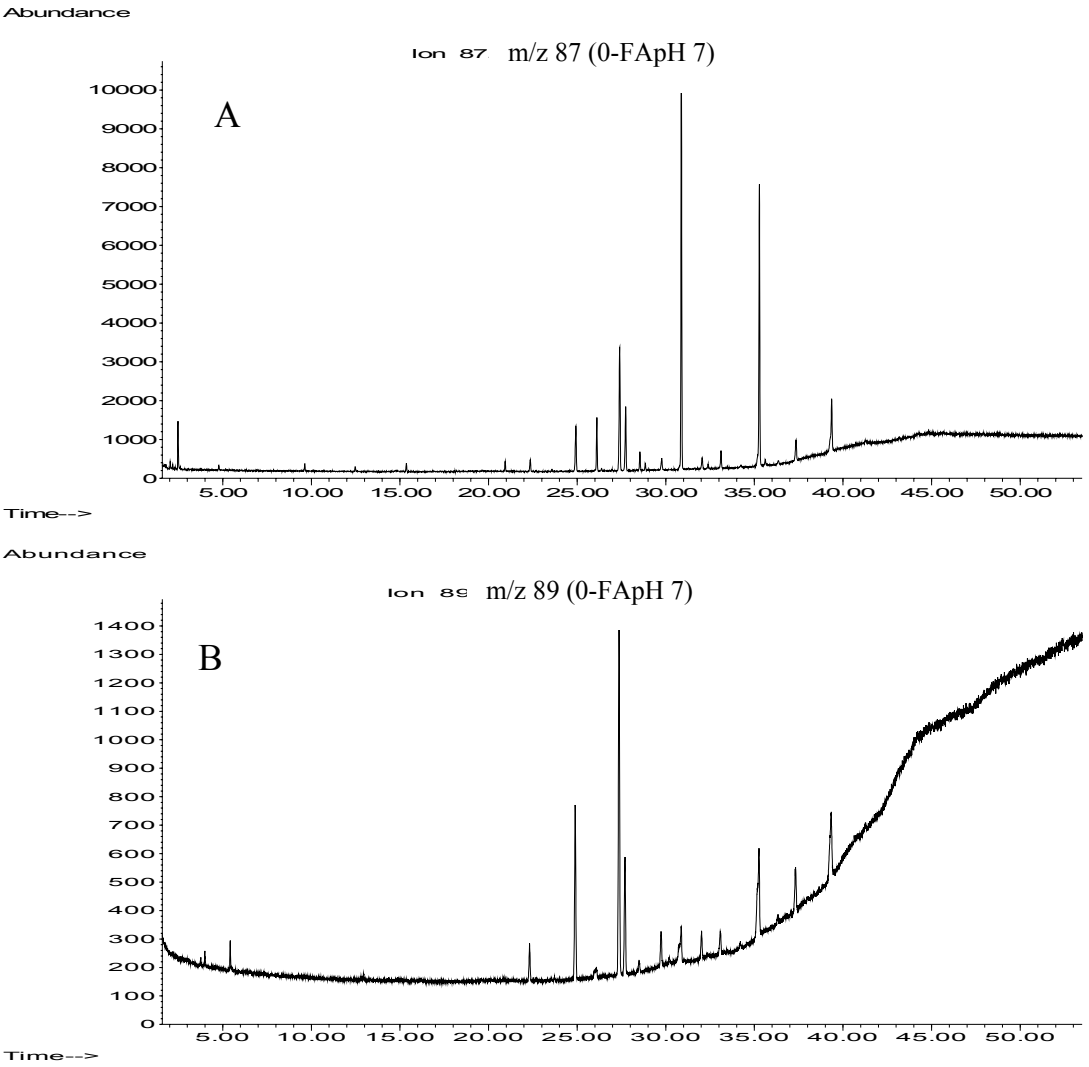
methyl 9,12,15-octadecatrienoate (α-linolenate or 18:3(*n*-3))



Appendix VI-2 Example of ion spectra of *S. aureus* cell fatty acid methyl esters obtained after growing at 37°C for 24h in minimal medium without of 1-^{13}C-2-d_3 sodium acetate and exogenous free fatty acids. A- m/z 87 ion spectra B- m/z 89 ion spectra



Appendix VI-3 Example of ion spectra of *S. aureus* cell fatty acid methyl esters obtained after growing at 37°C for 24h in minimal medium containing 1-^{13}C-2-d_3 sodium acetate and without exogenous free fatty acids. A- m/z 87 ion spectra B- m/z 89 ion spectra



VI- 7 References chapter VI

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CHAPTER VII

GENERAL CONCLUSIONS

The nutritional aspect of food comes after safety in the consumers purchase decision tree. The relation between polyunsaturated fatty acids (PUFA) and the prevention of pathologies like cardiovascular diseases and hypertension is widely established in literature. Increasing the polyunsaturated fatty acids of meat and especially of omega 3 fatty acids is another way of enhancing the quality of consumers diets. Introducing linseed in the rabbit fodder up to 6% of the diet increases the PUFA content of their meat. During storage, the release of free fatty acids (FFAs) in particular of polyunsaturated free fatty acids (PUFFAs) is carried out by microbial and meat endogenous enzymes, while the fatty acids oxidation under refrigerated temperature is mostly influenced by the microbial activity. According to the microbial growth and head space volatiles compounds, it can be hypothesized that the microflora is mostly influenced by the storage atmosphere and to a low extend by the meat fatty acid composition at refrigerated temperature.

The availability of FFAs, mostly composed of PUFFAs have different implications in the spoilage pattern of the minced meat. Lactic acid and psychrotrophic bacteria seem to be stimulated in their growth in the presence of PUFFAs particularly when the atmosphere of storage strengthens the environmental conditions. On the order side, PUFFAs can also contribute to the product safety by delaying or inhibiting the growth of some bacteria, in particular Gram-positive ones. These antibacterial properties of FFAs are reported in literature for a large number of food born bacteria. In this thesis, a more detailed approach with respect to the current literature was used, and consisted in taking into account some of the environmental variability in the assessment of the fatty acids inactivation potentialities. Food and food environmental parameters like pH, Aw, temperature can affect the bacterial response to antimicrobials and also the real availability of the antimicrobial itself. The cell fatty acid of *S. aureus* for example at 37°C has a high percentage of straight chain fatty acids when grown at pH 5 with respect to pH 7. This cell fatty acid variation at pH 5 lower the membrane fluidity and permeability, because of the increased packing capacity of the molecules. It is then expected that the antimicrobial activity will not have the same magnitude of action at different environmental pH values, also considering the influence of this parameter on the proportion of the fatty acid active form. The temperature also in consideration to the influence that have on the microbial response as well as the physical properties of the FFAs. We observed a high

probability of inactivation of the fatty acids when the cells were grown at high temperature with respect to refrigerated ones. Apart from the temperature and pH, the cell load also influence the probability of inactivation of free fatty acids. The probability of inhibition of *S. aureus* increased with the reduction of the inoculum cell load, and we did not observed any direct proportionality between MIC and cell load even though the MIC decreased with the inoculum concentration. When the available fatty acids is not enough to cause the death of the cells, the growth dynamics may be affected and in particular the growth parameters (lag time and growth rate). At low concentrations, the fatty acids tested in this thesis had very low influence on the growth rate of *S. aureus*, while the lag time was more affected. The single cell approach used to study the influence of the FFAs on the lag time allowed a detailed analysis of their effect on this phase of the *S. aureus* growth. This type of investigation associated to a stochastic birth process simulation can give an important information during the study of antimicrobial compounds. In the presence of free fatty acids concentrations, lower than the assessed MIC for a defined microrganism, the more the cell load is lower, the higher is the lag time and the variability associated to the lag time. These bactericidal and bacteriostatic properties of the free fatty acids differ in magnitude and rate, in function of the type of FFA. Within the FFA tested, C18:3 and C12:0 were the most active ones, while C10:0 and C8:0 had almost the same activity towards *S. aureus*. On the order hand, C18:0 and C18:1 did not show any inhibition potential up to 1500 ppm in the conditions tested, while C18:2 was active only in few conditions.

Physical properties and the modality of action can be regarded as the reasons behind the different amplitudes of inactivation observed with the selected FFAs tested against *S. aureus*. In fact, these fatty acids have different degrees of solubility and pKa, which influence their availability and partition into the cell membrane. In this thesis, we also investigated the influence of exogenous FFA on the synthesis of new cell fatty acids as a means of understanding their mechanism of action and also because of the scarcity of this type of information in literature. All the fatty acids tested influenced the fatty acid *de novo* synthesis in *S. aureus* even though in different ways while C18:3 was the only one which clearly inhibited the fatty acid biosynthesis elongation pathway. At pH 5, the *de novo* synthesis was quantitatively and qualitatively reduced with respect to pH 7 indicating the increased cell adaptation difficulties at that pH as a consequence of the enhanced fatty acid antimicrobial activity.

Coming back to the initial question, it can be said that respect to the conventional animal feeding, the inclusion of linseed in the animal diet enhances the fat nutritional quality, while the microbial safety related to the released FFAs depends more on the storage temperature and product

pH. The inhibitory potentials of the released FFAs will be probably more effective in a cured meat than in a fresh minced meat stored in refrigerated conditions. However, a multidisciplinary approach is needed to further understand the mechanism of action of free fatty acids, and their real availability in food products, in order to have a complete view on their antimicrobial properties in foods.