# ANTIBACTERIAL FREE FATTY ACIDS: ACTIVITIES, MECHANISMS OF ACTION AND BIOTECHNOLOGICAL POTENTIAL

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## Abstract

Amongst the diverse and potent biological activities of free fatty acids (FFAs) is the ability to kill or inhibit the growth of bacteria. The antibacterial properties of FFAs are used by many organisms to defend against parasitic or pathogenic bacteria. Whilst their antibacterial mode of action is still poorly understood, the prime target of FFA action is the cell membrane. Here, FFAs disrupt the electron transport chain and oxidative phosphorylation. Besides interfering with cellular energy production, FFA action may also result from the inhibition of enzyme activity, impairment of nutrient uptake, generation of toxic peroxidation and auto-oxidation degradation products or direct lysis of bacterial cells. Their broad spectrum of activity, non-specific mode of action and safety makes them attractive as antibacterial agents for various applications in medicine, agriculture and food preservation, especially where the use of conventional antibiotics is undesirable or prohibited. Moreover, the evolution of inducible FFA-resistant phenotypes is less problematic than with conventional antibiotics. The potential for commercial or biomedical exploitation of antibacterial FFAs, especially for those from natural sources, is discussed.

**Keywords:** antibiotic; antimicrobial; drug resistance; lipid; natural products.

## 1. Introduction

Fatty acids (FAs) are ubiquitous molecules typically found bound to other compounds such as glycerol, sugars or phosphate headgroups to form lipids. Lipids are integral components of cell structures, e.g. membranes, which are made up of phospholipids,

1 and energy stores that are often composed of triglycerides. FAs can be released from
2 lipids, typically by enzyme action, to become free fatty acids (FFAs), which have
3 diverse and potent biological activities (Table 1).
4
5 FFAs consist of a chain of carbon atoms attached to hydrogen atoms (Figure 1). The
6 number of carbon atoms varies but those in biological systems usually have an even
7 number between 10 and 28 and this review mainly concentrates on these. At one end
8 of the carbon chain is a carboxyl group (–COOH) and at the other end is a methyl
9 group (–$CH_3$) (Figure 1). The carboxyl group is hydrophilic and ionised when
10 solubilised in water whereas the carbon chain is hydrophobic, making the entire
11 molecule amphipathic. FAs with <8 carbon atoms are considered short-chain whereas
12 those with >16 carbon atoms are regarded as long-chain. Unsaturated FAs have one or
13 more C=C double bonds in the carbon chain while the carbon atoms in saturated FAs
14 are all joined by C–C single bonds (Figure 1). Lipases (the group of lipolytic enzymes
15 that cleave FAs from lipid headgroups by hydrolysis to give FFAs) can be specific for
16 certain types of lipid but they may also discriminate the FAs that they cleave on their
17 position on the lipid headgroup and the length and unsaturation of the FA's carbon
18 chain.
19
20 The biological activities of FFAs have roles in host defences against potential
21 pathogenic or opportunistic micro-organisms. An important aspect of this is growth
22 inhibition or the direct killing of bacteria. There is now an extensive literature
23 concerning the antibacterial effects of various FFAs from a wide range of biological
24 sources, including algae, animals and plants (McGaw et al. 2002; Wille and
25 Kydonieus 2003; Desbois et al. 2008, 2009). Indeed, FFAs are often identified as the

1 active ingredients in ethnic and herbal medicines (Yff et al. 2002; McGaw et al.
2 2002). This review aims to summarise some of this work and to discuss the various
3 mechanisms and structural features of FFAs that causes them to prevent bacterial
4 growth or survival. Furthermore, the potential for commercial or biomedical
5 exploitation of antibacterial FFAs is discussed.
6
7 **2. Free fatty acids in antibacterial defence**
8
9 The antibacterial effects of FFAs are frequently observed during bioassay-guided
10 fractionation of extracts from a variety of organisms (Hemsworth and Kochan 1978;
11 McGaw et al. 2002; Wille and Kydonieus 2003; Desbois et al. 2009). The
12 antibacterial actions of FFAs are typically broad spectrum and of potencies
13 comparable to natural antimicrobial peptides (AMPs) *in vitro* (Georgel et al. 2005).
14 FFAs function in the antimicrobial defences of many multicellular organisms,
15 including mammals (Hemsworth and Kochan 1978; Georgel et al. 2005), plants
16 (Weber 2002), molluscs (Benkendorff et al. 2005), seaweeds (Küpper et al. 2006) and
17 amphibians (Rickrode 1986). Whilst FFAs are not as structurally diverse as the more
18 widely studied AMPs their importance in the human innate immune system is well
19 established, particularly in the defence of skin and mucosal surfaces (Thormar and
20 Hilmarsson 2007; Drake et al. 2008). Indeed, FFAs are the most active antimicrobial
21 agents present in human skin lipid samples (Wille and Kydonieus 2003). There is 10-
22 15 µg of FFAs per square centimetre on human skin, of which lauric acid (C12:0),
23 myristic acid (C14:0), palmitic acid (C16:0), sapienic acid (C16:1n-10) and *cis*-8-
24 octadecenoic acid (C18:1n-10) are the most abundant (Wille and Kydonieus 2003;
25 Takigawa et al. 2005). FFAs are produced on the skin by lipolytic cleavage of lipids

secreted from the sebaceous glands (Shalita 1974; Fluhr et al. 2001; Drake et al. 2008) and their presence on the skin is sufficient to control the bacterial microbiota (Takigawa et al. 2005; Georgel et al. 2005; Kenny et al. 2009). The most important antibacterial FFA in human skin exudate is C16:1n-10, a long-chain monounsaturated FFA, and skin deficient in this and other FFAs tends to be more susceptible to colonisation by the opportunistic pathogen, *Staphylococcus aureus* (Takigawa et al 2005; Georgel et al. 2005). However, if the skin is treated with C16:1n-10 protection against colonisation is bolstered (Takigawa et al 2005; Georgel et al. 2005). Besides inhibiting or killing bacteria directly, FFAs also make conditions unfavourable for the growth of certain bacteria on the skin surface by maintaining an acidic pH (Fluhr et al. 2001; Takigawa et al. 2005). FFAs may further affect the expression of bacterial virulence factors (Table 1), which are important or essential for the establishment of an infection, probably by disrupting cell-to-cell signalling. Thus, saturated and unsaturated FFAs can prevent initial bacterial adhesion and subsequent biofilm formation (Kurihara et al 1999; Osawa et al 2001; Kankaanpää et al. 2004; Won et al 2007; Stenz et al. 2008; Davies and Marques 2009). Moreover, the swarming behaviour of the urinary tract pathogen, *Proteus mirabilis*, is inhibited by medium- and long-chain saturated FFAs (Liaw et al. 2004). The expression of certain toxins, haemolysins and enzymes conferring drug resistance are all down-regulated in the presence of various saturated and unsaturated FFAs (Ruzin and Novick 2000; Liaw et al. 2004; Clarke et al 2007) while genes responsible for iron uptake and extracellular proteases can be similarly reduced (Kenny et al 2009). The ability of various species of bacteria to resist the action of FFAs and subvert these epithelial defences certainly explains, at least in part, the success of certain skin and mucosal pathogens (Clarke et al. 2007; Drake et al. 2008).

1

2 Perhaps less well known is the role that FFAs play in the defence of single-celled
3 eukaryotic organisms against bacterial threats. In microbial eukaryotes, such as
4 microalgae, FAs are found primarily in the lipids that constitute the cell membranes
5 and energy storage structures but during cellular disintegration large quantities of
6 FFAs are released from cellular lipids by host lipolytic enzymes (Jüttner 2001;
7 Wichard et al. 2007). A high proportion of the FFAs that are freed from the cell
8 membranes, including those around the photosynthetic plastid, are mono- and
9 polyunsaturated varieties (Cutignano et al. 2006). These FFAs are toxic to
10 invertebrate grazers, which may have caused the microalgal cell to lose its integrity in
11 the first instance (Jüttner 2001; Wichard et al. 2007). Therefore, the toxic FFAs act to
12 reduce grazer numbers and ultimately grazing pressure (Jüttner 2001). At first it might
13 seem counter-intuitive that this defence strategy requires the host cell to undergo
14 mechanical damage and death but in evolutionary terms it has benefits because
15 neighbouring microalgal cells, particularly in biofilms, would be expected to be
16 clones or very closely related. That these same FFAs are potently antimicrobial means
17 similar protection may be afforded to microalgae under threat from pathogenic
18 bacteria or viruses. Whilst the initial host will not survive, FFAs released from a
19 microalgal cell that has been damaged by a pathogen will act on pathogens in the local
20 vicinity reducing their numbers, therefore conferring some protection of its
21 neighbouring relatives from onward transmission. This 'population level' defence
22 may be considered metabolically inexpensive as the FFAs form essential cellular
23 components with the lipases already synthesised and present within the cell to carry
24 out vital processes.

25

1 **3. Antibacterial activity and FFA structure**

2

3 The antibacterial activity of each FFA is influenced by its structure and shape. This, in
4 turn, is a function of the length of the carbon chain and the presence, number, position
5 and orientation of double bonds (Figure 2). The literature contains contrasting reports
6 concerning the relationship between a FFA's structure and its antibacterial activity but
7 some general trends do emerge. The –OH group of the carboxyl group seems to be
8 important for the antibacterial activity of FFAs as methylated FFAs (Figure 1) often
9 have reduced or no activity (Kodicek and Worden 1945; Zheng et al. 2005).

10

11 Medium- and long-chain unsaturated FFAs tend to be more active against Gram-
12 positive bacteria than Gram-negatives (Kodicek and Worden 1945; Galbraith et al.
13 1971). In general, unsaturated FFAs tend to have greater potency than saturated FFAs
14 with the same length carbon chain (Kabara et al. 1972; Greenway and Dyke 1979;
15 Feldlaufer et al. 1993; Zheng et al. 2005; Desbois et al. 2008). Within series of
16 monounsaturated FFAs, the most potent usually have 14 or 16 carbon atoms (Kabara
17 et al. 1972; Feldlaufer et al. 1993). Often a direct correlation exists between the
18 number of double bonds in an unsaturated FFA's carbon chain and its antibacterial
19 efficacy (Saito et al. 1984; Knapp and Melly 1986; Feldlaufer et al. 1993). The double
20 bonds in naturally occurring FFAs typically have *cis* orientation and these tend to
21 have greater antibacterial activity than FFAs with double bonds in *trans* orientation
22 (Galbraith et al. 1971; Kabara et al. 1972; Feldlaufer et al. 1993), probably because
23 the structures of *trans*-bonded unsaturated FFAs resemble saturated FFAs (Figure 2).
24 Whilst only a few studies have investigated the effect of bond position in the carbon
25 chain of FFAs there is some evidence that the position of double bonds can affect

1 potency and spectrum of antibacterial action (Kabara et al. 1977; Feldlaufer et al.
2 1993; Wille and Kydonieus 2003).
3
4 For saturated FFAs, the most active have 10 or 12 carbons in the chain and
5 antibacterial efficacy tends to decrease, as the chain length gets longer or shorter
6 (Galbraith et al. 1971; Kabara et al. 1972; Bergsson et al. 2001; Sun et al. 2003; Wille
7 and Kydonieus 2003). However, others workers have reported that FFAs with 14, 16
8 or 18 carbon atoms can be more potent than FFAs with 10 or 12 carbons against
9 certain species of bacteria (Willett and Morse 1966; Galbraith and Miller 1973a;
10 Miller et al. 1977). Comparisons between studies are complicated because different
11 authors have used a variety of methodological approaches to determine and measure
12 potency with considerable variation in the inoculum and incubation conditions.
13 Moreover, the relative activity of FFAs may depend on whether a complete growth
14 inhibition assay or an $IC_{50}$ determination is used (Willett and Morse 1966). To enable
15 simple comparison, ideally all determinations of minimum inhibitory concentration
16 (MIC) and minimum bactericidal concentration (MBC) for FFAs need to adhere to
17 standardised definitions and protocols such as those published by the Clinical and
18 Laboratory Standards Institute (CLSI) (CLSI, 2000).
19
20 **4. Mechanisms of antibacterial activity**
21
22 It remains unclear exactly how FFAs exert their antibacterial activities but the prime
23 target seems to be the bacterial cell membrane and the various essential processes that
24 occur within and at the membrane (Figure 3). Some of the detrimental effects on
25 bacterial cells can be attributed to the detergent properties of FFAs on account of their

1 amphipathic structures. This allows them to interact with the cell membrane to create
2 transient or permanent pores of variable size. At higher concentrations detergents,
3 such as FFAs, can solubilise the membrane to such an extent that various membrane
4 proteins or larger sections of the lipid bilayer are released. The key membrane-located
5 process affected by FFAs is the production of energy caused by interference with the
6 electron transport chain and the disruption of oxidative phosphorylation (Sheu and
7 Freese 1972; Galbraith and Miller 1973b; Miller et al. 1977; Boyaval et al. 1995;
8 Wojtczak and Więckowski 1999). Other processes that may contribute to bacterial
9 growth inhibition or death include: cell lysis, inhibition of enzyme activity,
10 impairment of nutrient uptake and the generation of toxic peroxidation and auto-
11 oxidation products (Figure 3). FFAs can kill a bacterium outright (bactericidal action)
12 or inhibit its growth (bacteriostatic action), which is reversible and means that the
13 bacterium remains viable but cannot undergo cell division in the presence of the FFA
14 (Kodicek and Worden 1945; Sheu and Freese 1972). Assays used to investigate the
15 antibacterial activities of FFAs do not always discriminate between bactericidal and
16 bacteriostatic actions but it is reasonable to assume that growth inhibition cannot
17 continue indefinitely and eventually a growth-inhibited bacterium will die. In
18 describing the processes of antibacterial activity below, little distinction is made as to
19 whether the outcome is bactericidal or bacteriostatic.
20
21 *4.1 Disruption of electron transport chain*
22 The inner membrane of Gram-positive and Gram-negative bacteria is an important
23 site for energy production and it is where the electron transport chain is located. The
24 various carriers in the electron transport chain, which are embedded within the
25 membrane, pass electrons from one carrier to another until two electrons combine

with the final acceptor, usually oxygen, and two protons to form water (Mitchell 1961). During this process protons are exported from the inside of the cell whilst the concentration of electrons in the cytosol increases. This generates a proton gradient and membrane potential which are crucial for the production of ATP by the enzyme, ATP synthase (Mitchell 1961). Medium- and long-chain saturated and unsaturated FFAs that gain access through the cell wall or outer membrane of a bacterium, can perhaps bind to the carriers of the electron transport chain directly or insert into the inner membrane causing the electron carriers to move apart or be displaced from the membrane entirely (Galbraith and Miller 1973b; Peters and Chin 2003). In each case the ability of the electron transport chain to transfer electrons is impaired so that the proton gradient and membrane potential are reduced. This results in a reduction in ATP production and the bacterium becomes deprived of an essential source of energy. Both unsaturated and saturated FFAs could translocate or bind the electron carriers directly but complete displacement from the membrane is likely achieved by unsaturated FFAs only, probably because they increase membrane fluidity (Greenway and Dyke 1979; Chamberlain et al. 1991; Stulnig et al. 2001). This is because the *cis*-bonds in unsaturated FAs cause a kink in the carbon chain (Figure 2) that prevents these FAs from packing tightly in the membrane. Thus, when medium- and long-chain unsaturated FFAs are incorporated into the membrane there is an increase in fluidity that can develop into membrane instability (Chamberlain et al. 1991; Stulnig et al. 2001). Conversely, medium- and long-chain saturated FFAs (and *trans*-bonded unsaturated FFAs) that lack a kinked structure can be packed more tightly (Figure 2). Hence, medium- and long-chain saturated FFAs can reduce membrane fluidity and disrupt electron transport perhaps by restricting the movement of carriers within the membrane (Sheu and Freese 1972). Moreover, as explained above, solubilisation of

1 the membrane by the detergent effect of FFAs could also account for the loss of vital
2 components of the electron transport chain from the membrane.
3
4 *4.2. Uncoupling of oxidative phosphorylation*
5 FFAs may further prevent energy production by uncoupling oxidative
6 phosphorylation. Thus the potential energy created by the electron transport chain
7 dissipates as heat rather than being used for ATP synthesis (Sheu and Freese 1972;
8 Greenway and Dyke 1979; Beck et al. 2007). ATP synthase (also located on the
9 bacterial inner membrane) uses the energy from the proton motive force, which results
10 from the proton gradient and membrane potential, created by the electron transport
11 chain, to convert ADP to ATP. Interaction of FFAs with the bacterial inner membrane
12 can affect this process and reduce or prevent the production of ATP (Sheu and Freese
13 1972; Galbraith and Miller 1973b). A simple way this could happen is for a saturated
14 or unsaturated FFA to bind directly to ATP synthase itself, which could prevent the
15 enzyme functioning correctly. Alternatively, FFAs can interfere with the proton
16 gradient and membrane potential. This weakens the proton motive force upon which
17 ATP synthesis relies. FFAs, particularly unsaturated ones, can reduce ATP synthesis
18 by increasing the permeability of the membrane to protons (Borst et al. 1962; Boyaval
19 et al. 1995). This could happen anywhere on the inner membrane or at specific proton
20 pores, such as those already identified in mitochondria (Więckowski and Wojtczak
21 1998; Wojtczak and Więckowski 1999; Beck et al. 2007). Thus protons enter the
22 cytosol causing a reduction in the proton gradient and membrane potential. Moreover,
23 the enzyme's ability to synthesise ATP is further diminished because the protons
24 bypass ATP synthase (Boyaval et al. 1995; Więckowski and Wojtczak 1998). The
25 proton gradient and membrane potential are also thought to be reduced by FFAs

entering the cytosol, dissociating the proton from its carboxyl group and then returning across the membrane to the exterior thus increasing the cytosolic concentration of protons (Wojtczak and Więckowski 1999; Beck et al. 2007; Schönfeld and Wojtczak 2008).

*4.3 Cell lysis*

Due to their structure, the insertion of unsaturated FFAs into the bacterial inner membrane causes it to become more fluid and permeable (Greenway and Dyke 1979; Chamberlain et al. 1991). The increased permeability of the membrane by the insertion of unsaturated medium- and long-chain FFAs can allow internal contents to leak from the cell, which can cause growth inhibition or even death (Galbraith and Miller 1973a; Greenway and Dyke 1979; Speert et al. 1979; Wang and Johnson 1992; Boyaval et al. 1995; Shin et al. 2007). If membrane fluidity increases excessively the membrane can become unstable and the cell will ultimately lyse (Carson and Daneo-Moore 1980). Indeed, unsaturated FFAs have been shown to lyse single-celled algae (Wu et al. 2006), bacteria (Carson and Daneo-Moore 1980; Wang and Johnson 1992; Thompson et al. 1994; Shin et al. 2007), erythrocytes (Fu et al. 2004), mammalian cells such as sheep fibroblasts (Thormar at el. 1987) and vero cells (Thormar at el. 1987), or even enveloped viruses (Thormar at el. 1987). Aside from increased membrane fluidity the detergent effect of FFAs, which at high concentrations may solubilise large sections of the cell membrane, could further account for complete cell lysis. In addition, saturated FFAs can induce autolysis of bacterial cell walls in some species, perhaps triggered by a reduction in membrane fluidity (Tsuchido et al. 1985; Cybulski et al. 2002; Kenny et al. 2009).

1 *4.4. Inhibition of enzyme activity*

2 FFAs are potent inhibitors of diverse enzymes and unsaturated FFAs usually have
3 greater inhibitory activity than saturated ones (Kurihara et al. 1999; Zheng et al. 2005;
4 Won et al. 2007; Hamel 2009; Sado-Kamdem et al. 2009). Inhibition of enzymes in
5 the membrane or cytosol that are crucial for bacterial survival and growth could
6 account for some of the antibacterial effects of FFAs. Interestingly, Zheng et al.
7 (2005) showed that unsaturated FFAs can inhibit bacterial fatty acid biosynthesis *in*
8 *vivo*, which will, in turn, affect the composition of the bacterial cell membrane. This
9 could cause altered and inappropriate cell membrane fluidity and permeability leading
10 to the membrane-related problems described above.
11
12 *4.5. Impairment of nutrient uptake*

13 Saturated and unsaturated FFAs can inhibit the ability of bacteria to take up nutrients,
14 such as amino acids, thereby effectively starving the bacterium of the nutrients it
15 requires to remain viable (Galbraith and Miller 1973b; Shibasaki and Kato 1978). It is
16 not clear whether FFAs reduce nutrient uptake by directly disrupting the membrane-
17 located transporter proteins (by direct binding or complete displacement) or whether it
18 is a consequence of the reduced proton motive force required for the energy-requiring
19 process of active transport.
20
21 *4.6 Peroxidation and auto-oxidation*

22 Other workers have suggested that it is the action of secondary degradation products
23 of FFAs that are responsible for their antibacterial activities. These could be produced
24 by peroxidation that yields $H_2O_2$ and reactive oxygen species (Knapp and Melly 1986;
25 Hazell and Graham 1990; Wang and Johnson 1992; Schönfeld and Wojtczak 2008) or

auto-oxidation of unsaturated FFAs that creates oxylipins and short-chain aldehydes, which are antibacterial in their own right (Gutteridge et al. 1974; Adolph et al. 2004).

Of course, the specific mechanisms by which individual FFAs cause bacterial growth inhibition and/or death will depend on fatty acid structure, the target bacterium and the sites that the FFA can access. Effective control of bacterial growth and survival might involve multiple mechanisms, each of which might, directly or indirectly, be affected by factors such as pH and temperature (Galbraith and Miller 1973c; Kabara et al. 1977; Miller et al. 1977; Shibasaki and Kato 1978; Greenway and Dyke 1979; Wang and Johnson 1992; Sun et al. 2003). Often it is not clear whether changes in antibacterial activity caused by different pH and temperature conditions is due to alterations in the solubility of the FFA or whether these conditions have greater influence on the physiology, and therefore the susceptibility, of the target bacterium.

## 5. Bacterial resistance to killing by FFAs

Some bacterial species are naturally resistant to the antibacterial action of FFAs. The cell walls of Gram-positive bacteria and the outer cell membranes of Gram-negative species protect against FFAs, as once these structures are removed the cells are more susceptible (Galbraith and Miller 1973a; Miller et al. 1977). Differential susceptibility of bacterial species to the action of FFAs is likely to be due to the FFA's ability to permeate the outer membrane or cell wall, which will enable access to the sites of action on the inner membrane. Interestingly, *S. aureus* appears to up-regulate the expression of genes encoding proteins involved in the synthesis of the cell wall upon exposure to unsaturated FFAs; a strategy that no doubt serves as a protective measure

because a thicker cell wall makes it more difficult for FFAs to penetrate and exert their antibacterial effects at the cell membrane (Kenny et al. 2009). Furthermore, additional wall material makes the cell surface more highly charged and thus less hydrophobic. Therefore FFAs are less attracted to the cell and are less likely to insert into the inner membrane (Clarke et al., 2007; Kenny et al. 2009). The ability of some bacteria to change their cell surface hydrophobicity (Clarke et al., 2007; Kenny et al. 2009) may explain why certain strains of the same species differ with respect to their susceptibility to the antibacterial effects of FFAs (e.g. Heczko et al. 1979; Ko et al. 1978; Lacey and Lord 1981; Kenny et al. 2009).

Another factor that might contribute to the resistance of some bacterial strains to disruption by FFA is the presence of membrane-located carotenoids. Carotenoids are antioxidants that also stabilise the cell membrane by decreasing its fluidity. Thus their presence may counteract the effects of reactive FFA degradation products or FFA-induced increases in membrane fluidity (Chamberlain et al. 1991). Indeed, strains of *S. aureus* containing high levels of carotenoids are less susceptible to the antibacterial effects of unsaturated FFAs than strains with lower quantities of carotenoids in their membranes (Chamberlain et al. 1991; Xiong and Kapral 1992). Further work is necessary to ascertain whether similar differences in the presence of carotenoids, or other membrane-stabilising sterols, account for the variation in FFA susceptibility between different strains of other bacterial species. Certainly there is a need to better elucidate the precise mechanism(s) of antibacterial action by FFAs in order to understand how certain bacteria evade or abrogate their bactericidal effects.

## 5. Uses and applications of antibacterial free fatty acids

The broad spectrum of activity and non-specific mode of action of at least some FFAs make them attractive as antibacterial agents for various applications in medicine, agriculture, food preservation and the formulation of cosmetics or nutraceuticals, especially where the use of conventional antibiotics is undesirable or forbidden. Many FFAs are plentiful in natural sources, non-toxic (Kabara 1979) and 'generally regarded as safe' (U.S. Food and Drug Administration 2007). By and large, the evolution of inducible FFA-resistant phenotypes is less problematic than with conventional antibiotics (Lacey and Lord 1981; Petschow et al. 1996; Sun et al. 2003). Kenny et al. (2009) screened 5000 transposon mutants of *S. aureus* for FFA-resistance but found none and, in fact, most mutants were even more susceptible to FFA action. Yet despite their obvious potential, the antibacterial properties of FFAs have still to be fully exploited. One reason may be because some FFAs, particularly long-chain polyunsaturated ones, can be unstable (Kodicek and Worden 1945; Gutteridge et al. 1974; Guil-Guerrero et al. 2001) and tend to bind non-specifically to proteins or other compounds (Kodicek and Worden 1945; Galbraith et al. 1971; Lacey and Lord 1981; Boyaval et al. 1995; Petschow et al. 1996). A further problem may be the perceived lack of patentable intellectual property concerning these ubiquitous antibacterial compounds. However, these problems can be overcome and the usefulness of FFAs in antibacterial applications should not be dismissed.

*5.1 Biomedical therapeutics*

FFAs are defence molecules in the innate immune systems of multicellular organisms that could be manipulated for the prevention and treatment of bacterial diseases. The increasing prevalence of drug-resistant bacteria as well as an enhanced appreciation

1 for the mechanisms of drug-resistance acquisition is necessitating the discovery and
2 development of alternative anti-infectives to conventional antibiotics (Thormar and
3 Hilmarsson 2007). The future exploitation of particular FFAs for systemic treatment
4 may be limited by their toxicity at high doses to certain eukaryotic cells (Table 1),
5 although Clarke et al. (2007) successfully used C16:1n-10 to treat systemic *S. aureus*
6 infections in mice. At present, the best prospects for exploitation in medicine are for
7 therapies aimed at enhancing the concentrations of natural FFAs on the skin.
8
9 Topical antibacterial decolonising agents are given to patients intra-nasally before
10 surgery to disinfect the nose and reduce the chances of contracting a post-surgical
11 infection (van Rijen et al. 2008). Presently, the antibiotic of choice for this is
12 mupirocin but resistance to this agent is becoming increasingly prevalent and
13 treatment failure is now more common (Simor et al. 2007). Linolenic acid (C18:3) can
14 reduce *S. aureus* numbers on human skin and therefore could be exploited as an
15 alternative to mupirocin (Lacey and Lord 1981). Furthermore, Lukowski et al. (2008)
16 have shown that emulsions of fatty acid-rich extracts from microalgae can reduce
17 MRSA attachment to pre-treated skin. Thus, there is potential for the development of
18 a gel containing one or more FFAs with potent activity for Gram-positive pathogens,
19 such as MRSA, to prevent and reduce bacterial colonisation of the skin and nose.
20
21 As far as sexual health is concerned there are also possibilities for a FFA-containing
22 product to reduce the transmission of sexually transmitted infections (STIs),
23 especially those caused by *Neisseria gonorrhoeae* (Bergsson et al. 1999; Thormar et
24 al. 1999), *Chlamydia trachomatis* (Bergsson et al. 1998; Thormar et al. 1999) or
25 herpes simplex virus (Kristmundsdóttir et al 1999). Indeed, formulations containing

monoglycerides (single FAs bound to glycerol) have demonstrable efficacy against STIs *in vivo* (Neyts et al. 2000). Other potential therapeutic applications suggested for FFAs in humans might be in the prevention of dental caries (Kurihara et al. 1999; Osawa et al. 2001; Won et al. 2007), in reducing the incidence of infant gastrointestinal infections by adding to formula milk (Thormar et al. 1987; Isaacs et al. 1995), in the treatment of acne (Nakatsuji et al 2009; Yang et al 2009), or in the treatment of stomach ulcers caused by *Helicobacter pylori* (Hazell and Graham 1990; Thompson et al. 1994; Petschow et al. 1996).

*5.2 Agriculture and aquaculture*

Antibiotics are used as animal feed supplements to increase the production of meat or cultured fish, as they reduce bacterial abundance in the digestive system resulting in more energy being diverted to weight accumulation (food conversion) (Dibner and Richards 2005). However, concerns about antibiotic resistance transferring to human pathogens and anxiety about antibiotic residues and environmental contamination (Smith et al. 2002) has led to the ban on the use of conventional antibiotics in livestock foodstuffs in the European Union (European Union, 2005) and similar bans are being considered elsewhere (Dibner and Richards 2005). Therefore, opportunities exist to replace these conventional antimicrobial agents and FFAs may be a realistic alternative. Moreover, as FFAs are also active against methane producing Archaea (methanogens) in the guts of ruminants they could reduce emissions of this important greenhouse gas (Ungerfeld et al. 2005).

Piglets treated with a source of lipids and a lipolytic enzyme to release antibacterial FFAs in the animals' guts show a reduction in the abundance of gut microbiota and

1 improved weight gain and feed conversion (Dierick et al. 2002). However, at present,
2 the high cost of the lipid component remains the stumbling block to implementation
3 (Dierick et al. 2002). Here, we suggest that single-celled algae could be an
4 inexpensive source of FFAs. These micro-organisms are autotrophic, negating the
5 need for costly heterotrophic sources of carbon, can be cultured on non-arable land in
6 salt water and can achieve growth rates similar to bacteria (de la Noue and De Pauw
7 1988). Moreover, technologies in the culture, harvest and manipulation of single-
8 celled algae for their lipids are established but continue to improve, particularly due to
9 recent interest in biofuels (Chisti 2008). Single-celled algal species can be selected
10 and their lipid composition further manipulated to enrich for the particular mixture of
11 FFAs required (Borowitzka 1988). The algae, which are a nutrient source in
12 themselves, typically also contain various health promoting vitamins and antioxidants
13 (de la Noue and De Pauw 1988), could be incorporated into animal feed. The addition
14 of exogenous lipolytic enzymes may be unnecessary for certain algal species as the
15 cells contain their own enzymes activated on cell disintegration (Jüttner 2001;
16 Wichard et al. 2007).
17
18 FFAs added to feed may also increase survival in commercial rabbit farms where
19 losses to enteric diseases can be high. Rabbits experimentally infected with
20 pathogenic *Escherichia coli* have a better chance of survival if the feed is
21 supplemented with caprylic acid in its free form or as triglycerides (Skřivanová et al.
22 2008). These rabbits also have significantly fewer *E. coli* in their faeces and stomachs
23 compared to rabbits fed a non-supplemented diet (Skřivanová et al. 2008). Emulsions
24 of monoglycerides can reduce the burden of pathogenic bacteria, such as
25 *Campylobacter jejuni*, in chicken feed (Thormar et al. 2006). A similar approach

could be used for the prevention of disease in aquaculture and mariculture, as FFAs are active against industry-relevant bacterial pathogens (Benkendorff et al. 2005; Desbois et al. 2009) and pose virtually no environmental harm from leaching into the water. Finally, antibacterial FFAs have also been considered in the treatment of bovine mastitis (Hogan et al. 1987; Nair et al. 2005) and in the control of honeybee infections (Feldlaufer et al. 1993; Hornitzky 2003).

## 6. Discussion and concluding remarks

As discussed above, the antibacterial properties of FFAs are well recognised and because they act through different mechanisms to most conventional antibiotics they offer potential for commercial exploitation. However, there are a few problems that have hindered progress thus far. First, some FFAs have an unpleasant taste (Stephan and Steinhart 2000; Refsgaard et al. 2000). Second, certain FFAs can be unstable and they also have a tendency to bind non-specifically to proteins (Kodicek and Worden 1945; Galbraith et al. 1971; Lacey and Lord 1981; Boyaval et al. 1995; Petschow et al. 1996; Guil-Guerrero et al. 2001). Finally, there may be a perceived lack of patentable intellectual property (IP) because FFAs are found so ubiquitously. As regards taste, a possible solution is to deliver the FFAs in the form of lipids together with a lipolytic enzyme. Such a combination, where the enzyme cleaves antibacterial FFAs from the lipid source, can be used to increase the *in situ* abundance of FFAs, such as inside an animal’s gut (Dierick et al. 2002). This form of administration also subverts the problem of FFA instability because the FFAs will be delivered as stable lipids (e.g. triglycerides). If the problem of taste can be solved, one of the most lucrative areas for development could be in controlling the growth of pathogens or

spoilage bacteria in food (Wang and Johnson 1992; Ouattara et al. 1997; Shin et al. 2007; Desbois et al. 2008, 2009). Monoglycerides, which tend not to have an unsavoury taste, are already used in food preservation (Shibasaki and Kato 1978). One of the most exciting potential applications for antibacterial FFAs is their use in topical medicine for the prevention and treatment of bacterial diseases. With respect to IP opportunities, new IP could be generated by exploring interactions between FFAs and conventional antibiotics or other agents with the aim of identifying synergistic combinations, as investigations to this end have been reported only sparsely (Shibasaki and Kato 1978; Wille and Kydonieus 2003; Drake et al. 2008). Combination therapies, where multiple antibacterial agents are given together, are desirable as they can reduce the opportunity for bacterial resistance to emerge (Zhao and Drlica 2001). Treatments containing a FFA component will further reduce the opportunity for resistance to emerge due to the FFA's non-specific mode of action. Additionally, studies of chemically altered FFAs engineered for more desirable 'drug-like' characteristics may prove to be another fruitful avenue to success.

Ultimately, the choice of FFAs is application-dependent and will differ according to the requirements of the process and the bacteria to be targeted. Specific mixtures could be produced that are optimised for each application and finely tuned for potency and spectrum. These could be mixed with solvents, stabilisers or other compounds to further enhance activity.

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1 **Figure legends**

2

3 **Figure 1.** Structure of free fatty acids (FFAs). (A) The saturated FFA, capric acid
4 (C10:0), which has 10 carbon atoms in the carbon chain. The carbon chain length can
5 vary but at one end is the carboxyl group (–COOH) while at the other end is a methyl
6 group ($–CH_3$). The carboxyl group is hydrophilic and ionised when solubilised in
7 water whereas the carbon chain and terminal methyl group are hydrophobic, making
8 the entire molecule amphipathic. (B) Unsaturated FFAs have one or more C=C double
9 bonds in the carbon chain and, here, the fatty acid is methylated, as the carboxyl
10 group has an additional $–CH_2$. This fatty acid is C10:2n-3 as there are 10 carbon
11 atoms in the carbon chain, there are 2 C=C bonds of which the first of these is located
12 3 carbon-carbon bonds from the methyl end.

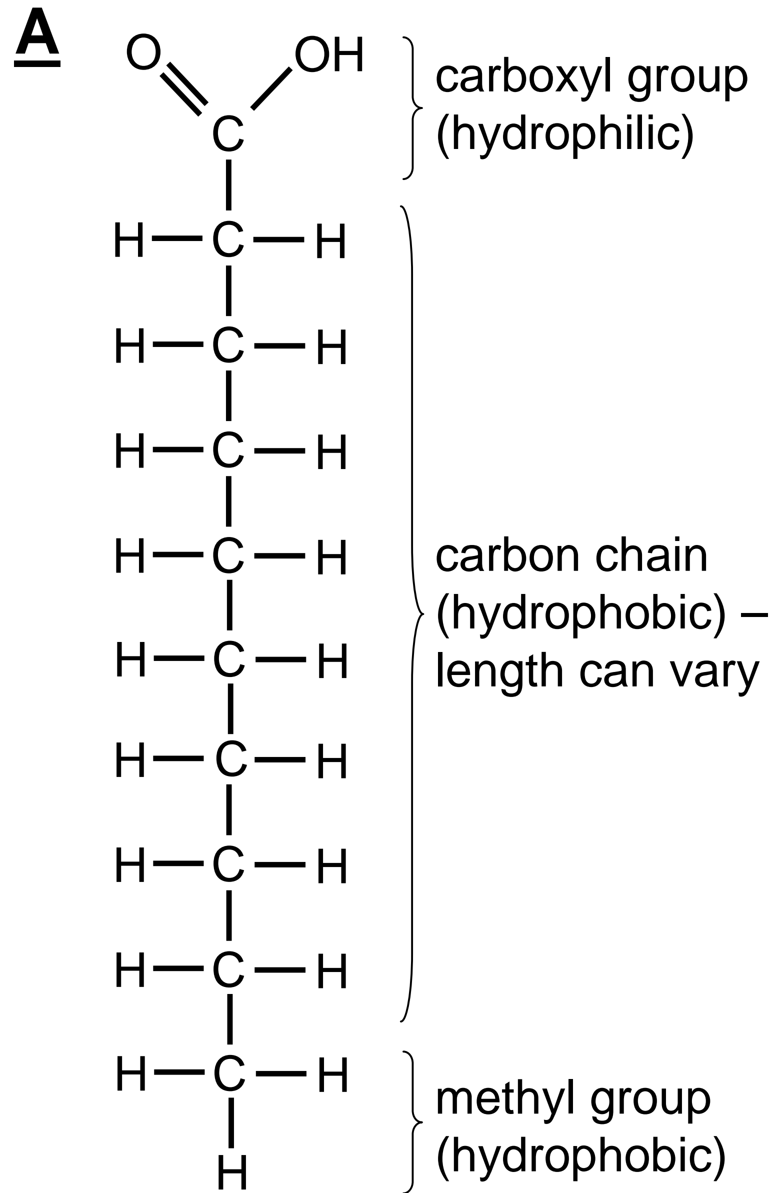
13

14 **Figure 2.** Space-filled representations of 8 free fatty acids (FFAs). Saturated FFAs
15 (e.g. lauric and stearic acids) have a simple 'straight-line' structure. A *cis*-double bond
16 causes a kink in the carbon chain (e.g. myristic and oleic acids). Additional *cis*-double
17 bonds in the carbon chain cause further kinks (e.g. linoleic, γ-linolenic and
18 eicosapentaenoic acids). However, *trans*-double bonds have little effect on the shape
19 (e.g. elaidic acid) and these FFAs structurally tend to resemble saturated FFAs (e.g.
20 stearic acid).

21

22 **Figure 3.** Schematic representation of possible cell targets and mechanisms of
23 antibacterial activity of free fatty acids (FFAs). They may affect bacterial energy
24 production by disrupting the electron transport chain and/or interfering with oxidative
25 phosphorylation. FFAs can cause leakage of cell metabolites from the cell, complete

1 cell lysis and autolysis. Membrane and cytosolic enzymes, including those required
2 for fatty acid biosynthesis, can be inhibited by FFAs. They can impair active nutrient
3 uptake by acting directly on the transport protein or as an indirect result of the cell's
4 inability to produce ATP. Peroxidation and auto-oxidation products of FFAs may also
5 have deleterious effects on the bacterial cell and play a role in cell killing. For clarity,
6 only the bacterial inner cell membrane is shown.
7



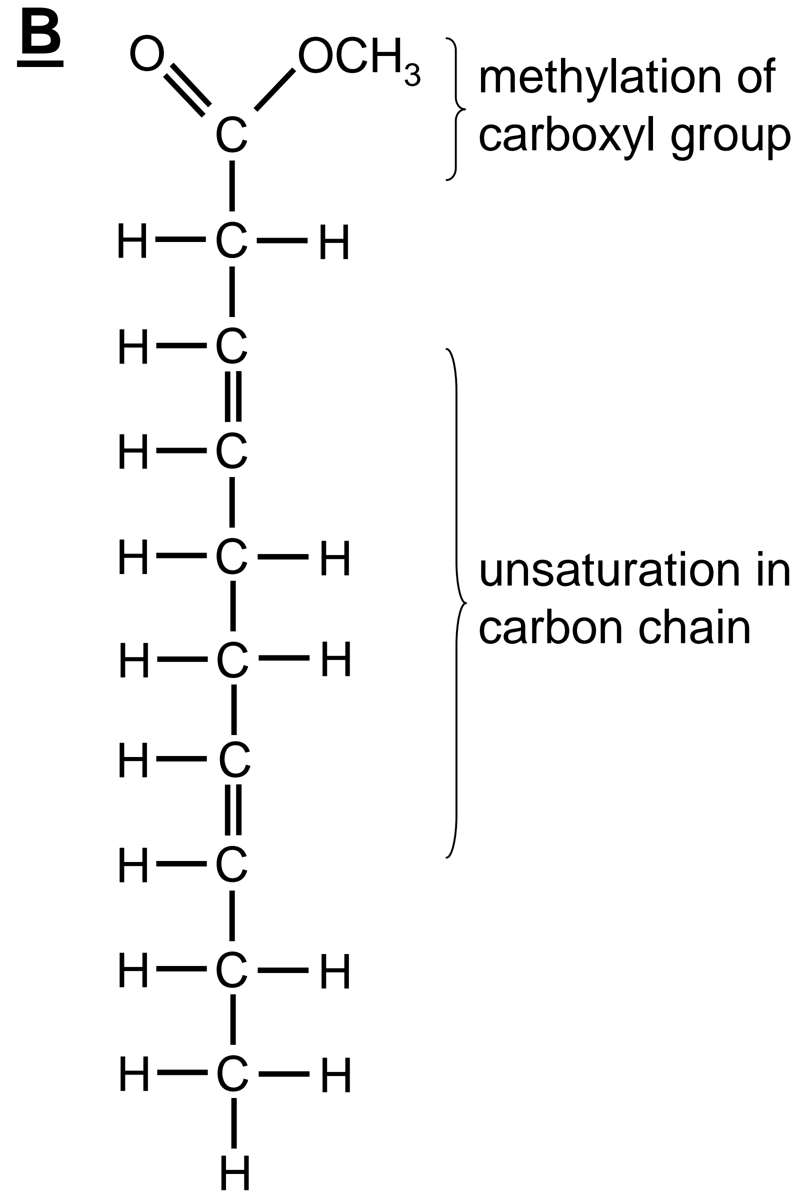
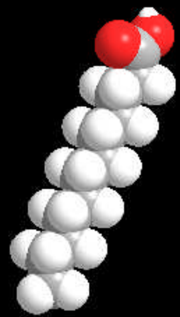
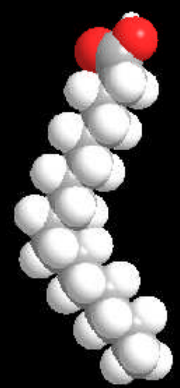




Fig. 1

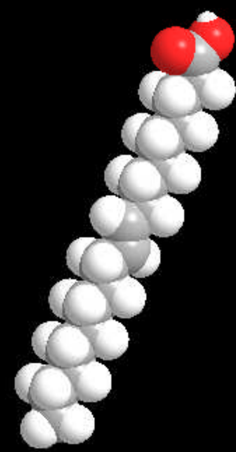
Fig. 2



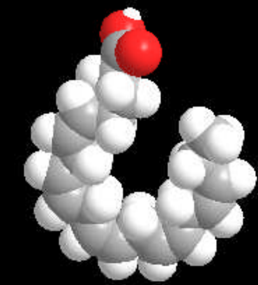
lauric acid (C12:0)



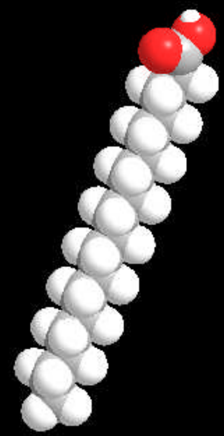
myristoleic acid (C14:1n5)



elaidic acid (C18:1n-9*t*)



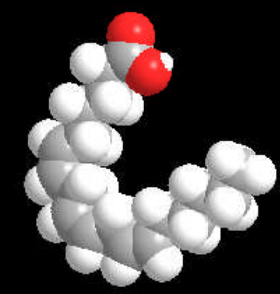
eicosapentaenoic acid (C20:5n-3)



stearic acid (C18:0)

oleic acid (C18:1n-9)

linoleic acid (C18:2n-6)



γ-linolenic acid (C18:3n-6)

**Disruption of electron transport chain by:**

- direct binding to electron carriers.
- insertion between carriers preventing their interaction.
- complete displacement of carriers from the membrane.
- preventing carrier interactions by reducing fluidity of the membrane.

**Interference with oxidative phosphorylation by:**

- preventing correct functioning of ATP synthase by direct binding or complete displacement from the membrane.
- reducing proton gradient/membrane potential by increasing membrane permeability to protons or FFAs dissociating a proton inside the cell and then returning to the extracellular space.

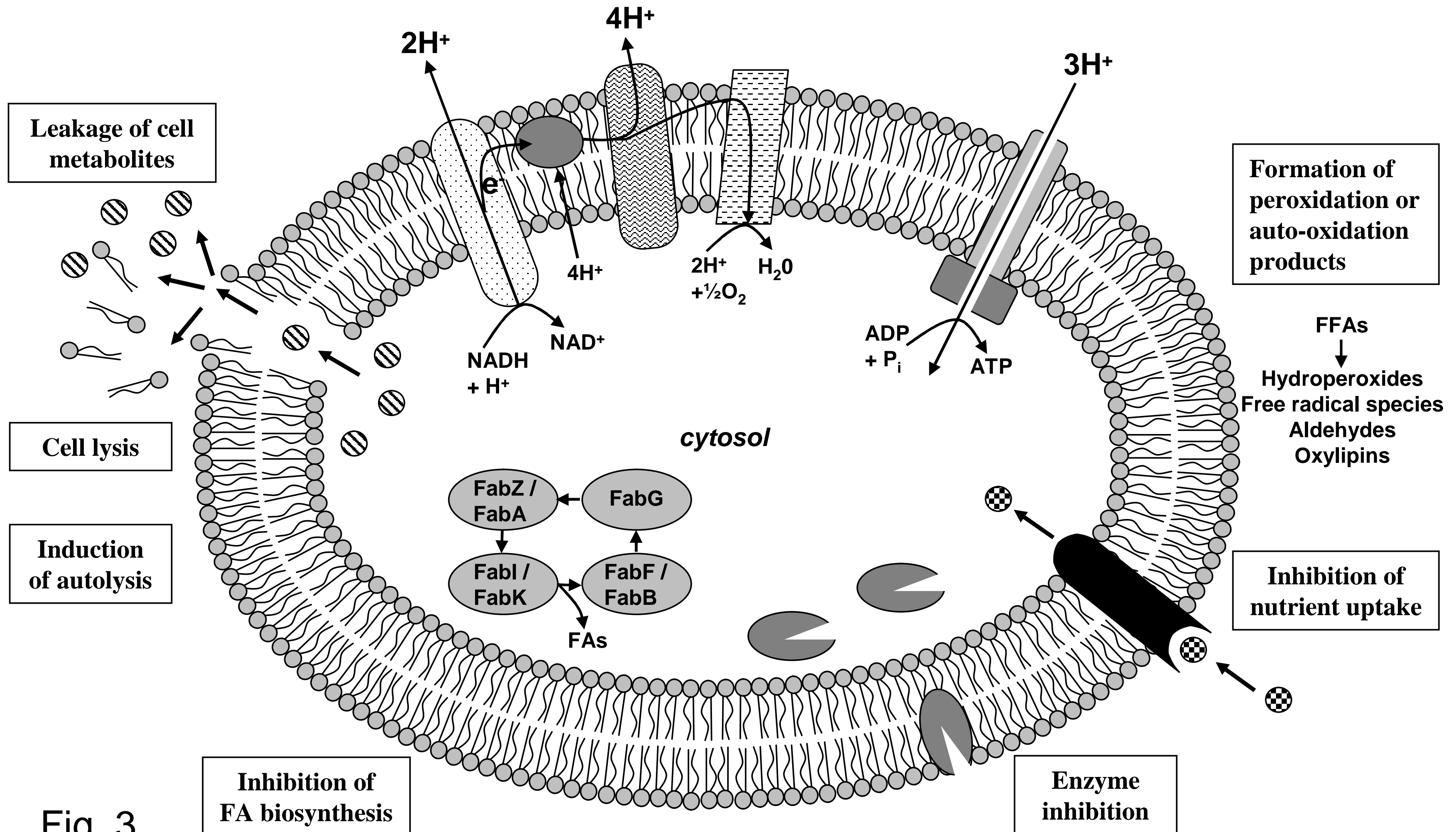



Fig. 3

**Table 1 –** Selected bioactivites of various saturated and unsaturated FFAs. Bond positions, where reported, are all in *cis* orientation unless marked *t* for *trans*.

| Activity | Fatty acid(s) | Reference |
|---|---|---|
| *Antimicrobial* | | |
| Anti-algal | C8:0, C10:0, C12:0 | McGrattan et al. (1976) |
| | C18:4n-3 | Kakisawa et al. (1988) |
| | C18:1, C18:2, C18:4, C18:5, C20:4, C20:5, C22:6 | Arzul et al. (1995) |
| | C18:2n-6, C18:3n-3 | Ikawa et al. (1997) |
| | C16:0, C18:0, C18:1n-9, C18:2, C18:3n-3, C20:5n-3, C22:6n-3 | Wu et al. (2006) |
| | C16:0, C16:1n-7, C16:1n-7*t*, C16:4n-3, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, C18:4n-3, C20:0, C20:1n-9, C20:4n-6, C20:5n-3, C22:0, C22:1n-9, C22:6n-3 | Alamsjah et al. (2008) |
| Antibacterial (Gram-negative) | C20:4n-6 | Knapp and Melly (1986) |
| | C10:0, C12:0 | Bergsson et al. (1998) |
| | C10:0, C12:0, C14:0, C16:1 | Bergsson et al. (1999) |
| | C15:0, C16:0, C17:0, C18:0, C18:1, C18:4, C20:4, C20:5, C22:0, C22:4, C22:5 | Benkendorff et al. (2005) |
| Antibacterial (Gram-positive) | C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3 | Galbraith et al. (1971) |
| | C10:0, C12:0, C14:0, C14:1, C16:0, C16:1, C18:1, C18:2, C18:3 | Kabara et al. (1972) |
| | C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1n-5, C16:1n-7, C16:1n-7*t*, C18:2n-6, C18:3n-3, C18:3n-6, C20:1n-9, C20:3n-6, C20:3n-3, C20:4n-6, C22:2n-6, C22:3n-3, C20:4n-6, C22:6n-3 | Feldlaufer et al. (1993) |

| | | |
|---|---|---|
| | C16:1n-10 | Wille and Kydonieus (2003) |
| | C15:0, C18:1, C18:4, C20:4, C20:5, C22:0, C22:4, C22:5 | Benkendorff et al. (2005) |
| Anti-fungal | C10:0, C12:0 | Bergsson et al. (2001) |
| | C10:0, C12:0, C14:0, C14:1, C16:1, C18:2 | Kabara et al. (1972) |
| Anti-protozoan | C18:0, C18:1, C18:2, C18:3 | Rohrer et al. (1986) |
| | C8:0, C10:0, C12:0 | Dohme et al. (2001) |
| Antiviral | C8:0, C10:0, C12:0, C14:0, C16:1, C18:1, C18:2, C18:3, C20:4 | Thormar et al. (1987) |
| | C10:0, C12:0, C14:0, C16:1, C18:1 | Hilmarsson et al. (2006) |
| *Cytotoxic* | | |
| Haemolytic (sheep erythrocytes) | C18:0, C18:1, C18:2, C18:3, C18:4, C18:5, C20:4, C20:5, C22:6 | Arzul et al. (1995) |
| Haemolytic (human erythrocytes) | C20:4n-6, C20:5n-3 | Fu et al. (2004) |
| Inhibits cell division (mammalian leukemic HL-60 cells) | C20:4n-6, C20:5n-3, C22:6n-3 | Finstad et al. (1994) |
| Inhibits cell division (sea urchin eggs) | C18:4, C18:5, C20:5, C22:6 | Sellem et al. (2000) |
| Inhibits development of fertilised echinoderm eggs | C16:4n-3 | Murakami et al. (1989) |
| Inhibits photosynthesis | C16:1n-7 | Peters and Chin (2003) |
| Reduces viability of rat Leydig cells | C16:0, C18:0 | Lu et al. (2003) |
| *Toxic to whole organisms* | | |

| | | |
|---|---|---|
| Brine shrimp larvae | C8:0, C10:0, C12:0, C18:1, C18:2, C18:3, C20:4 | Curtis et al. (1974) |
| *Daphnia* (Crustacean) | C18:3n-6 | Reinikainen et al. (2001) |
| Fairy shrimp (Crustacean) | C20:5n-3 | Jüttner (2001) |
| Fish | C20:5n-3 | Marshall et al. (2003) |
| Mosquito larvae | C18:1, C18:2, C18:3n-6 | Harada et al. (2000) |
| Tube worm (marine) | C20:4, C20:5 | Pawlik (1986) |
| *Signalling* | | |
| Increases expression of bacterial proteins for energy metabolism, cell wall and protein synthesis | C16:1n-6, C18:2n-6 | Kenny et al. (2009) |
| Induces larval settlement and metamorphosis | C16:1, C18:2, C20:4, C20:5 | Pawlik (1986); Jensen et al. (1990) |
| Inhibits bacterial attachment | C18:1n-9 | Stenz et al. (2008) |
| Reduces expression of bacterial virulence factors: β-lactamase and Toxic Shock Syndrome Toxin (TSST) | C12:0 | Ruzin and Novick (2000) |
| Reduces expression of bacterial virulence factors: β-lactamase and haemolysin | C16:1n-6 | Clarke et al. (2007) |
| Reduces expression of bacterial virulence factors: haemolysin | C12:0, C14:0, C16:0, C18:0 | Liaw et al. (2004) |
| Regulates bacterial swarming | C12:0, C14:0, C16:0, C18:0, C18:1 | Liaw et al. (2004) |
| Regulates protein kinase C activation | C20:4n-6 | Khan et al. (1995) |