



Amine-based compounds cause bacterial viability loss at alkaline pH

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“Life isn’t about waiting for the storm to pass,
it’s about learning to dance in the rain”

Vivian Greene

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List of abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BHI	brain heart infusion
CCCP	carbonyl cyanide <i>m</i> -chlorophenyl hydrazone
cf	carboxyfluorescein
cFDA	5(6)-carboxyfluorescein diacetate
CFU	colony forming unit
CLSI	clinical and laboratory standards institute
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
D-ala	D-alanine
DAPI	4'6-diamidino-2-phenylindole
D-glu	D-glutamate
DiBAC ₄	bis-(1,3-dibutylbarbituric acid)trimethine oxonol
DiOC ₂	3,3'-Diethyloxacarbocyanine iodide
DMEA	<i>N,N</i> -dimethylmonoethanolamine
DMG	<i>N,N</i> -dimethylglycine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAI	emulsifying activity index
EMA	ethidium monoazide
ESI	emulsion stability index
ESBL	extended spectrum β -lactamase
ETEC	enterotoxigenic <i>Escherichia coli</i>
FCM	flow cytometry
gly	glycine
L-ala	L-alanine
M-A ₂ pm	meso-diaminopimelic acid
MEA	monoethanolamine
MFI	mean fluorescence intensity
min	minutes
MIC	minimal inhibitory concentration
MMEA	<i>N</i> -monomethylethanolamine
mRNA	messenger ribonucleic acid
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
MurNAc	<i>N</i> -acetylmuramic acid

MWF	metalworking fluid
NAD	nicotinamide adenine dinucleotide
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PMF	proton motive force
polyP	inorganic polyphosphate
rATP	recombinant adenosine triphosphate
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
UV	ultra violet
VBNC	viable-but-non-culturable
VRE	vancomycin resistant enterococci

General Introduction



1 WHY IS THERE A NEED FOR ANTIMICROBIAL ALTERNATIVES?

1.1 Background

When antibiotics were first put to use in the early 20th century, they dramatically reduced morbidity and mortality due to bacterial infections in the industrialized world. However, in recent decades this progress has been tempered by the consistent increase in (multi)resistance towards these antimicrobials by certain microorganisms (Chastre, 2008). Many of the currently available classes of antibacterial compounds were developed between the 1940s and 1960s (Spellberg et al., 2004). Only a few new classes of antimicrobials have been described since. The vast majority of compounds introduced in recent years are modifications of known antimicrobial agents and the final registration rate of new effective antimicrobial drugs is surprisingly low (Barrett and Barrett, 2003).

When exposed to harmful conditions, bacteria will employ many ingenious mechanisms to survive (Russel, 2003). External stress caused by specific environmental conditions has various effects on different bacteria, leading to natural responses like inhibition and/or inactivation. Any deviation from the normal conditions might result in reduced growth rates. When bacteria are exposed to sublethal levels of biocides, only minor cell damage is caused. These changes in their phenotype and induction of gene expression can give rise to a more resistant population (Poole, 2012). Antimicrobial resistance is a major global public health problem. Given the adaptability of microorganisms and their tendency to acquire resistance, the development of innovative antimicrobial strategies has become a constant challenge.

The emerge of methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE) and extended spectrum β -lactamase (ESBL)-producing Enterobacteriaceae has dramatically reduced the number of empirical agents suitable for selected indications (Nobmann et al., 2009; Park et al., 2011). Many alternatives have been investigated to date. Antimicrobial peptides are considered as alternatives for conventional antimicrobial agents. These compounds often possess substantial antimicrobial activity and have the ability to broadly modulate immune activity when the host is invaded by pathogenic microorganisms (Zasloff, 2002). Surfactants are also of interest, more specifically amphoteric compounds, such as betaines and amine oxides. These have been shown to exhibit antimicrobial activity against various types of organisms (Corner et al., 1988; Lindstedt et al., 1990; Birnie et al., 2000). However, the environmental risk and the potential host toxicity are additional critical aspects to consider. Amino acid-based antimicrobials offer many advantages in this regard. They are biodegradable and typically possess low toxicity. The amino acid-derived compounds



used in this PhD thesis, i.e. glycine, *N*-methylglycine (sarcosine), *N,N*-dimethylglycine (DMG) and *N,N,N*-trimethylglycine (betaine) are therefore attractive candidates in the search for antimicrobial alternatives.

1.2 The veterinary perspective

The first goal of livestock production is the delivery of safe food for human consumption taking into account the welfare of the animal and respect for the environment. In the past, one of the strategies was to add antibiotics to the animal feed, mostly at sub-therapeutic levels, to act as growth promoters (de Lange et al., 2010). However, in 2006, the use of antibiotics as growth promoters was banned in the European Union due to major concerns about antimicrobial resistance development and potential residual contamination of the food chain (Vondruskova et al., 2010; Daudelin et al., 2011).

The exact mechanisms by which in-feed antibiotics influence animal growth are not completely understood. Their growth promoting effect may at least partially be attributed to the suppression of some pathogenic bacteria, such as enterotoxigenic *Escherichia coli* (ETEC, *E. coli*), and of related gut infections/growth-depressing toxins. It is also often stated that antibiotics influence the normal, non-pathogenic gut microflora. These changes will have a beneficial effect on digestive processes and the utilization of nutrients in feed (Visek, 1978; Gaskins et al., 2002). The ban on in-feed antibiotics has raised the need to look for sound alternatives that could enhance the natural defense mechanisms of animals and reduce the pathogenic load in the intestinal tract.

This can be achieved by using specific feed additives or dietary raw materials to favourably affect animal performance and welfare. A large number of feed additives have been evaluated to date. They are mostly aimed at (i) enhancing the animal's immune response (e.g. immunoglobulin, ω -3 fatty acids, β -glucans, oral vaccination), (ii) reducing the pathogenic load in the gut (e.g. organic and inorganic acids, zinc oxide, essential oils, herbs and spices, some types of prebiotics, bacteriophages, and antimicrobial peptides), (iii) stimulating the establishment of beneficial gut microbes (probiotics and some types of prebiotics), and (iv) stimulating digestive function (e.g. butyric acid, lactic acid, glutamine) (de Lange et al., 2010; Gaggia et al., 2010; Melkebeek et al., 2013).

The beneficial effects of amino acid-based compounds such as betaine and DMG in animal production have been suggested (Fernandez-Figares et al., 2002; Klasing et al., 2002; Eklund et al., 2005, 2006a,b; Metzler-Zebeli et al., 2009; Kalmar et al., 2010a,b). However, the antibacterial effects of these compounds on gut pathogens have not yet been described.



1.3 The industrial perspective

Biofilms are another important aspect in the emergence of antimicrobial resistance. They are defined as microbial communities adhering to various surfaces and embedded in a self-produced extracellular matrix (Høiby et al., 2010; Park et al., 2011). Biofilms are often associated with hospital infections due to their high resistance to antimicrobial compounds. In more recent years they have also been studied in the industrial sector. An industrial segment that suffers from such antimicrobial resistance problems is the metalworking industry (Lucchesi et al., 2012). Indeed, cutting fluids commonly used in metalworking are an ideal environment for bacterial growth. Among the contaminants commonly found in these fluids are *Pseudomonas* spp., *Escherichia coli*, and *Mycobacterium* spp. (Perkins and Angenent, 2010; Murat et al., 2011; Lucchesi et al., 2012; Saha and Donofrio, 2012). The control of contamination of metalworking fluids (MWF) is usually done by the addition of different antimicrobial agents (Rossmore, 1995; Marchand et al., 2010). Alkanolamines are used in MWF as corrosion inhibitors, but they also appear to have antimicrobial effects (Sandin et al., 1990; Bakalova et al., 2008). The underlying mechanism of these antibacterial effects has not yet been investigated in depth to date and we hypothesize it to be related to antibacterial effects we propose in this PhD thesis at first for glycine and its derivatives.

Assessment of bacterial viability after exposure to potentially antibacterial compounds provides information on both the antibacterial potential and the possible mechanism of action of these compounds.

2 HOW TO ASSESS BACTERIAL VIABILITY

2.1 Definition of bacterial viability

Ever since Van Leeuwenhoek first described bacteria (Porter, 1976), humans have tried to control and restrict their growth for a variety of reasons. The presence of bacteria is essential for certain processes, while in some situations the growth of bacteria has to be blocked completely. As a consequence, the ability to measure the viability of bacterial cells has a major role in microbiology. The question how to define bacterial viability however, remains a challenging task to date as there is no simple answer to it. Van Leeuwenhoek first equated viability with motility (Porter, 1976), but the definition and hence determination of bacterial viability turned out to be much more complex. Davey et al. (2004) state that a microbial cell is generally considered “viable” if it possesses all the components and mechanisms necessary for sustained proliferation, being a definition related to growth capacity. Some years before,



Breeuwer and Abee (2000) already emphasized the “survival” aspect, stating that viable cells are those cells capable of performing all functions necessary for survival under certain circumstances. Survival in this respect can be defined as the continuing existence of the species.

Literature offers many different opinions on the true meaning of bacterial viability. Knowing what is truly alive or dead and what are the possible intermediate states, remains one of the major challenges in microbiological analysis. Moreover, the ability to distinguish different physiological states is especially important for assessing the survival of pathogenic bacteria after exposure to certain antimicrobial agents. It offers information on either the bacteriostatic or the bactericidal potential of compounds and can aid in identifying their underlying working mechanisms (Bogosian & Bourneuf, 2001).

2.2 Viability versus Culturability

The original concept of viability assessment, introduced more than a century ago, was to elucidate the ability of bacteria to grow/reproduce (Allen et al., 2004). Traditional microbiology often uses dilution plating and manual cell counts as the gold standard for proof of cell viability, stating that a cell is viable when it has been shown to proliferate (Joux and Lebaron, 2000; Nebe-von-caron et al., 2000; Davey et al., 2004). However, this approach has several drawbacks which can lead to a substantial underestimation of both the bacterial viability and the actual number of viable bacteria present. Plate counting can leave sublethally damaged organisms, fastidious and uncultivable bacteria, and viable cells that have lost the ability to form colonies undetected under certain test conditions (Keer and Birch, 2003). A growth-based definition assumes that all viable cells are able to reproduce and does not take into account the nutrient requirement, the potential for long lag phases and the growth medium composition (Davey et al., 2004; Baatout et al., 2005). Moreover, some bacteria can revert between states of culturability and unculturability, the so-called “viable-but-not-culturable” (VBNC) states (Breeuwer and Abee, 2000; Darcan et al., 2009). This may lead to potential false negative results (Davey et al., 2004).

In general, the conflict on viability and culturability is somewhat paradoxical. On the one hand, traditional culture based methods rely on the dividing capacity of a cell, which requires at least a few hours and sometimes even a few days to be detected and carries a risk of underestimation. On the other hand, physiological probes which have been developed for real-time monitoring of cell viability do not allow the control of cell division (Joux and Lebaron, 2000). Therefore, viability analysis should be based on a combination of real-time physiological techniques and the standard culture-dependent methods, allowing the evaluation of viable and dead cells as well as many possible intermediate states.



2.3 Viable-But-Non-Culturable (VBNC)

The survival of bacteria under suboptimal conditions (“stress”) depends on their ability to regulate and control their metabolism in such a way that they are able to survive (Darcan et al., 2009). The term VBNC has been introduced during recent years for apparently viable bacterial cells which have become non-culturable due to exposure to such suboptimal circumstances. The VBNC state is thought to be a transient physiological state in which cells switch off activities typical for growing organisms, but maintain a low level of metabolic activity without being culturable on routine microbiological media (Oliver, 2010). There is a decrease in synthesis of macromolecules, in nutrient transport and in cell respiration, even cell morphology can change (Oliver, 2005; Smith and Oliver, 2006). Bacteria may enter the VBNC state due to various external stress factors such as cold stress, nutrient deficiency, osmotic shock, oxidative stress or UV stress (Oliver, 2005). These stress factors can cause bacterial damage, preventing immediate culturability or alternatively, trigger specific genetic survival mechanisms causing the bacteria to pass into a VBNC state (Bogosian & Bourneuf, 2001). These VBNC bacteria either remain in this non-culturable state until they are exposed to more beneficial conditions stimulating their resuscitation, or they gradually die (McDougals et al., 1998; Kell and Young, 2000; Oliver, 2005). Because these non-culturable cells still possess a distinct activity and may still be pathogenic, they should be denoted as viable (Oliver, 2010). Virulence is often retained by these bacteria, even as the ability to produce toxins, and infection can occur upon resuscitation to an active state (Oliver, 2010). Many bacteria in the environment are in the VBNC state and it should be mentioned that they, next to the negative threat associated with resuscitation of pathogenic species, also carry great potential for desirable environmental functions, more especially for their possible benefits in bioremediation (Su et al., 2013).

2.4 Determination of viability

Reproductive growth is considered to be the most stringent proof of viability, it requires both metabolic activity and membrane integrity (Nebe-von-Caron et al., 2000). Nevertheless, as stated above, in many cases growth itself cannot be measured due to irreversible DNA damage, fastidious growth requirements, lack of symbiotic partners or extremely slow growth. Detection of metabolic activity, which provides presumptive evidence of reproductive capacity, can be used as an alternative. However, in case of injury, dormancy or extreme starvation, metabolic function may be transiently undetectable (Nebe-von-Caron et al., 2000). In the absence of metabolic activity, membrane integrity can be determined as an indicator of viability. Indeed, both proliferation and metabolic activity depend on an intact cytoplasmic membrane. Cells with intact membranes are presumed capable of metabolic activity, repair and proliferation, unless their DNA is damaged beyond repair or unless they cannot



generate a positive energy balance (Nebe-von-Caron et al., 2000; Hammes et al., 2011). Next to the culture-based methods, several viability indicators can be assessed without the need to culture cells, and each method is based on criteria that reflect different levels of cellular integrity or functionality (Berney et al., 2006).

Gaining information on viability states of bacteria using fluorescent probes is of great interest nowadays and can be performed based on various cell characteristics. Indicators for cell viability can be activities of enzymes involved in substrate uptake and cleavage, the energy status of a cell and the integrity state of cell membranes (Berney et al., 2007; Sträuber and Müller, 2010). All these individual characteristics can be evaluated using complementary microscopic techniques and flow cytometry, which provide information on the physiological state of the bacteria at a single cell level.

However, caution should be taken as the interpretation of such viability data is often ambiguous. The use of only one viability indicator does not suffice to describe the physiological state of a bacterial cell under stress. Only the combined data from several complementary methods, including the detection of culturability, provides some level of certainty about the physiological state of a bacterium (Berney et al., 2006).

A concise descriptive list of the possible bacterial viability determination methods is given below.

2.4.1 Growth-based conventional methods

2.4.1.1 Viable cell counts on agar plates

Traditionally, the number of viable microorganisms is determined by simple plate counting. Briefly, a diluted sample is spread over a solid agar, followed by incubation under bacteria-specific optimal conditions allowing each viable organism to develop into a distinct colony on the plate. The initial number of viable organisms in the sample can be calculated from the number of colonies formed, multiplied by the initial dilution factor (Li et al., 1996). This method does not take into account viable but non-growing bacteria and thus provides no information on the actual state of the bacteria. It does however broadly reflect the antimicrobial potential of compounds tested.

2.4.1.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC value of an antibacterial compound is defined as the lowest concentration that is able to inhibit visible bacterial growth and thus provides only information on the growth capacity of the bacterial population. It is usually determined using one of the following methods: microdilution (in 96-well plates), macrodilution (in tubes) or agar dilution (also called plate dilution). These methods



are used to measure quantitatively the *in vitro* activity of an antimicrobial agent against a given bacterial isolate (Pankey and Sabath, 2004).

The MBC value on the other hand is the lowest concentration of an antimicrobial agent required to kill all bacteria (> 99.9%). It can be determined in parallel with MIC tests by subculturing the MIC samples on agar media without the antimicrobial agent and serves to differentiate bacteriostatic from bactericidal antimicrobial agents (Pankey and Sabath, 2004).

As the plate counts, MIC and MBC data again lack information on the physiological and metabolic state of the bacteria. Even though they are often considered to be gold standards for antimicrobial activity, these methods only imply population measurements and assume that bacterial populations are homogeneous, which is usually not the case. However, the information generated from these tests provides a good indication for the antimicrobial potential of the compounds under investigation.

2.4.1.3 Optical Density (OD) – Turbidity

An increase in both cell mass and cell number can be estimated by spectrophotometrical measurement of the turbidity of a bacterial suspension (Dalgaard and Koutsoumanis, 2001). The bacterial cells scatter light and presuming the cell size is constant, the amount of light scattered is proportional to the concentration of cells present in a sample. However, this traditional technique monitors bacterial proliferation and has the same disadvantages as the two previously described growth-based methods (Baatout et al., 2005). This method does not distinguish live bacteria from dead bacteria or even particles (Hazan et al., 2012).

2.4.2 Cultivation-independent viability analysis

Even though conventional culture-dependent methods are still of major importance for the routine assessment of bacterial viability and killing, several alternative methods are now available offering greater accuracy and specificity (Joux and Lebaron, 2000; Sträuber and Müller, 2010; Hammes et al., 2011). Next to the ability to grow and reproduce, bacteria possess various other measurable signs of viability, mainly based on their structural integrity and metabolic activity. Fig.1 provides a schematic overview on the complementary bacterial viability indicators. Based on the expected mode of action and the required application of an antimicrobial agent, it is advised to measure multiple viability parameters (Joux and Lebaron, 2000; Sträuber and Müller, 2010; Hammes et al., 2011).

Cultivation-independent viability analysis at the single cell level is one of the most attractive features of flow cytometry (FCM). Its outright advantage is that it allows rapid and quantitative viability analysis of unculturable bacteria (Shapiro, 2000; Berney et al., 2008), or normally culturable bacteria that are injured rendering them unable to grow (Lehtinen et al., 2006). Information on the single cell



level also provides information on the heterogeneity of a bacterial population (Shapiro, 2000). In addition, the availability of multiple fluorescent stains with different target sites facilitates the allocation of a sequence of damage effects to selected cellular compartments (Novo et al., 2000; Berney et al., 2006; Bosshard et al., 2009). This way, considerably more information is gained on the mode of cell damage and the function of bactericidal agents. Moreover, FCM viability analysis often identifies intermediate states, or so-called injured cells, during viability analysis, which allows to refine the merely “viable or not” distinction (Berney et al., 2007; Davey, 2011).

Flow cytometric analysis alone however is not sufficient to provide a straightforward answer to the viability question. It is imperative to combine such viability data with complementary viability parameters (Hammes and Egli, 2010). Where possible, direct culturability (Berney et al., 2006), ATP analysis (Berney et al., 2006, 2008; Hammes et al., 2008) and even labelled substrate incorporation (Servais et al., 2003) should be complementary determined to validate and support the flow cytometric data.

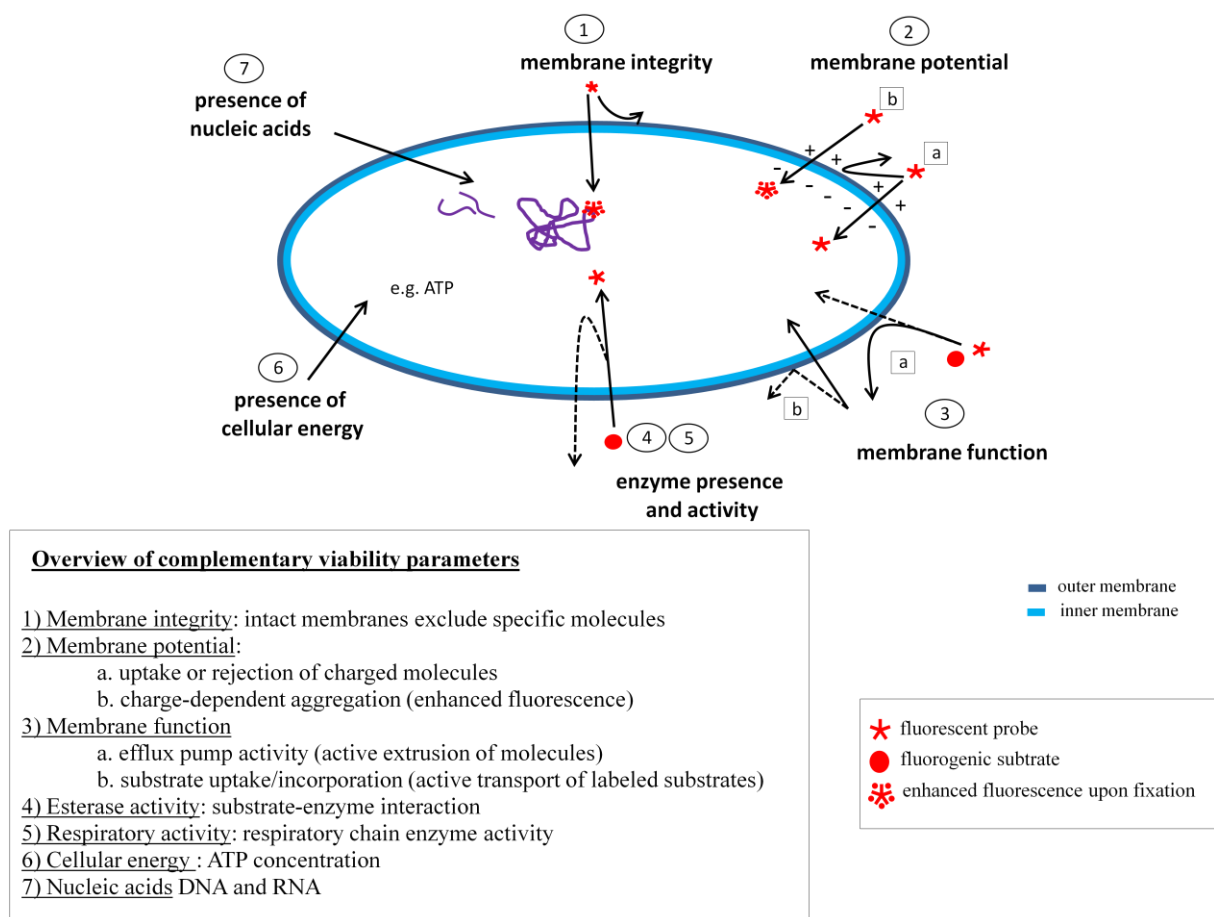


Figure 1: Overview of complementary viability parameters.
(Adapted from Joux and Lebaron, 2000; Hammes et al., 2011)



2.4.2.1 Membrane integrity

The cytoplasmic membrane of all microbial cells permits them to interact with the external environment. It is selective and permeable, determining what enters and leaves bacteria. An intact polarized cytoplasmic membrane is essential for a fully functional, healthy cell, and the impairment of membrane integrity disturbs several functions associated with the plasma membrane, such as respiratory activity, transport, permeability barrier and others (Baatout et al., 2005).

Membrane integrity analysis is based on the capacity of cells to exclude a fluorescent dye which, when used at low concentrations, does not normally cross intact membranes (Fig.1) (Jepras et al., 1995; Joux and Lebaron, 2000). Nucleic acids stains are often used for this purpose because of the high concentrations of nucleic acids within cells and the large fluorescence enhancement exhibited by these stains upon binding. The latter leads to a clear separation between intact and permeabilized cells (Joux and Lebaron, 2000; Baatout et al., 2005). Loss of membrane integrity as measured by uptake of membrane-impermeant dyes is generally considered to be irreversible. Nevertheless, restoration of the membrane integrity has also been reported (Votyakova et al., 1994).

Propidium iodide (PI) is a hydrophilic cationic molecule which can only passively pass through compromised membranes (Sträuber and Müller, 2010). It is widely used as a membrane integrity indicator for (microbial) cells and is known to selectively stain dead cells (Berney et al., 2007; Sträuber and Müller, 2010). However, some reports found that PI also labels highly productive cells. Shi et al. (2007) suggested that PI may enter through shortly open cell wall structures which arise during the elongation phase of bacterial cell division. Nevertheless, *E. coli* was found not to present elevated PI mean fluorescence intensity (MFI) values during growth experiments, indicating that this process does not occur in *E. coli* (Shi et al., 2007).

Several commercial single dyes and dye combinations are available allowing assessment of bacterial viability based on membrane integrity. In this PhD thesis the BacLight Live/Dead kit was used. It contains two nucleic acid stains (PI and SYTO9), differing in their spectral characteristics and in their ability to penetrate intact bacterial membranes. While PI only penetrates cells with damaged membranes, staining the cells red, SYTO9 enters cells with either intact or damaged membranes, staining them green. When both fluorochromes are combined, the emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and due to the quenching by fluorescence resonance energy transfer (Joux and Lebaron, 2000; Stocks, 2004; Berney et al., 2007). Next to these two defined states of viability (“live” green and “dead” red bacteria), “intermediate” states are also observed (Virta et al., 1998; Joux and Lebaron, 2000; Christiansen et al., 2003; Hoefel et al., 2003; Berney et al., 2006; Berney et al. 2007). These bacteria simultaneously display different degrees of green as well as red fluorescence. The latter patterns of fluorescence are directly related to the extent of the membrane damage present (Berney et al., 2007) and are illustrated in Fig. 2. When such a bacterial population initially shows a higher SYTO9 (green) fluorescence, but



not yet a PI (red) fluorescence, this is indicative for an increasing membrane permeability that permits the entry of more SYTO9 molecules, but still excludes the larger PI molecules (Berney et al., 2007). Only if the membrane damage has become more severe and thus potentially lethal, PI also enters the bacterial cell.

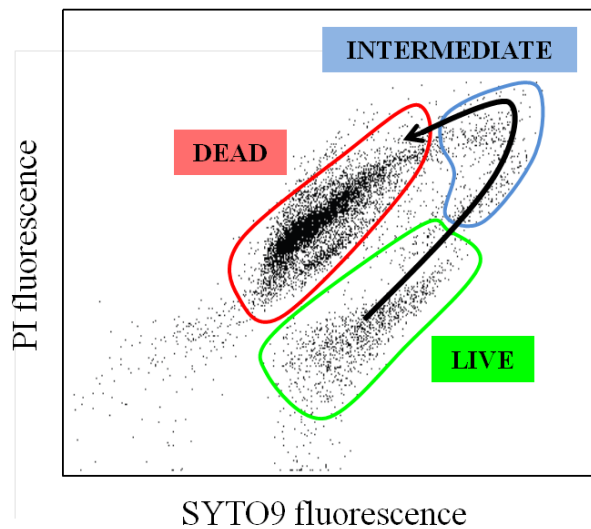


Figure 2: Flow cytometric SYTO9/PI dot plot presenting a population of *E. coli* at different stages of membrane damage. The arrow specifies changes in the position of various bacterial subpopulations moving from live over intermediate to dead

2.4.2.2 Membrane potential

The membrane potential ($\Delta\Psi$) of metabolizing bacteria is generated through respiration, ATP hydrolysis and concentration gradients of ions such as sodium, potassium, chloride and protons (Shapiro, 2000; Sträuber and Müller, 2010). The latter gradients result from the selective permeability of the cytoplasmic membrane to a variety of cations and anions. They are maintained and modulated by the action of various electrogenic pumps and ion channels (Joux and Lebaron, 2000). Bacteria normally maintain a $\Delta\Psi$ higher than 100 mV across their cytoplasmic membrane, with the interior side being negatively charged (Sträuber and Müller, 2010). The $\Delta\Psi$ thus plays a key role in physiological processes of bacterial cells: it is involved in ATP generation, active transport, bacterial chemotaxis and regulation of the intracellular pH. It also reflects membrane integrity and energy status, as well as cell viability (Novo et al., 1999; Shapiro, 2000; Baatout et al., 2005; Sträuber and Müller, 2010).

In general, bacterial cells only generate a sufficiently large $\Delta\Psi$ allowing proper staining when in high metabolizing phases such as exponential growth (Sträuber and Müller, 2010). Voltage-sensitive dyes are used to measure the $\Delta\Psi$ in bacteria. Reliability of this staining is confirmed by determination of dye uptake by bacterial cells treated with proton gradient uncouplers (e.g. carbonyl cyanide *m*-chlorophenyl hydrazone, CCCP) or ionophores (e.g. nigericin, valinomycin) (Joux and Lebaron, 2000).



In this PhD thesis the cationic carbocyanine dye DiOC₂ was used. This is a positively charged lipophilic fluorescent probe, which freely crosses the cytoplasmic membrane and only remains intracellular when a sufficient $\Delta\Psi$ is present (Baatout et al., 2005; Sträuber and Müller, 2010). DiOC₂ exhibits green fluorescence in all bacterial cells, but the fluorescence shifts towards red emission as the dye molecules aggregate at higher cytosolic concentrations caused by a larger $\Delta\Psi$. This red fluorescence depends on both $\Delta\Psi$ and cell size, while the green fluorescence is $\Delta\Psi$ -independent and only reflects cell size. The ratio of red to green fluorescence intensity thus provides a measure for $\Delta\Psi$ that is largely cell size-independent (Novo et al., 2000).

The $\Delta\Psi$ can also be determined by the anionic lipophilic oxonols. These dyes will only enter the bacterial cell when its $\Delta\Psi$ is reduced, concentrating and binding to lipid-rich components (Joux and Lebaron, 2000). They undergo a $\Delta\Psi$ -dependent distribution between the cytoplasmic membrane and the extracellular medium (Jepras et al., 1995). A much used example of this type of dye is 1,3-dibutylbarbituric acid (DiBAC₄). It has a high voltage sensitivity and emits a green fluorescence which is enhanced upon accumulation (Bränuer et al., 1984).

2.4.2.3 Membrane function

General membrane function is typically determined by the evaluation of efflux pump activity. This provides both structural (i.e. membrane integrity) and functional ($\Delta\Psi$) information (Hammes et al., 2011). Efflux pumps are used as a defensive measure to limit the intracellular concentration of a toxic compound and plays an important role in drug resistance (Marquez et al., 2005). Molecules that can passively cross an intact cytoplasmic membrane are pumped outside active cells by (non-)selective proton antiport systems. Bacterial efflux pumps can be divided into five families based on their structural characteristics, mechanism(s) of action and source of energy for the transport process: (i) the ATP-binding cassette (ABC) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the multidrug and toxic compound extrusion (MATE) family, (iv) the small multidrug resistance (SMR) family (a subgroup of the drug/metabolite transporter superfamily), and (v) the resistance-nodulation-division (RND) superfamily (Li and Nikaido, 2009). Efflux pump activity is usually assessed with ethidium bromide fluorescence. As ethidium bromide is a small molecule, it enters the cell through passive uptake. When efflux pump activity is lost, it accumulates intracellular and binds to nucleic acids (Bosshard et al., 2009; Wang et al., 2011). Ethidium bromide emits weak fluorescence in aqueous solutions and becomes strongly fluorescent when its intracellular concentration exceeds the external milieu concentration (Jernaes and Steen, 1994).



2.4.2.4 Enzymatic activity

Bacteria maintain a number of housekeeping enzymes with relative general functions (e.g. esterases, dehydrogenases and peptidases). In this PhD thesis, the demonstration of esterase activity was used to provide an indication of the bacterial metabolic activity. Like other housekeeping enzymes, these esterases illustrate the capacity of a cell to synthesize and maintain new proteins. However, although enzyme synthesis requires energy, it should be emphasized that the esterase-substrate reactions do not, and as such, these latter assays can be considered as energy-independent (Joux and Lebaron, 2000; Nebe-von-Caron et al., 2000; Baatout et al., 2005; Hammes et al., 2011).

Lipophilic, uncharged and non-fluorescent fluorogenic substrates, such as carboxyfluorescein diacetate (cFDA), are commonly used to determine esterase activity (Hoefel et al., 2003). These substrates are cleaved in active and intact cells by non-specific esterases, including lipase and acylase (but not acetylcholinesterase), and result in the release of fluorescent products like fluorescein or - derivatives. Being highly polar, the fluorescein(derivative) is trapped only within cells with an intact membrane. The fluorescence intensity will increase proportionally with the metabolic activity of the esterases (Joux and Lebaron, 2000; Baatout et al., 2005). The fluorogenic substrates for esterases thus serve as enzyme activity as well as membrane integrity probes (Baatout et al., 2005).

2.4.2.5 Respiratory activity

Respiratory activity in bacteria depends on a fully functional electron transport chain, which is the main process for maintaining the $\Delta\Psi$. This makes the presence of respiratory activity a good indicator for bacterial viability, linking $\Delta\Psi$ and the recycling of reducing equivalents (e.g. NAD^+) that are produced in catabolic reactions (Hammes et al., 2011).

In the cellular electron transport system, tetrazolium salts can function as artificial redox partners instead of the physiological final electron acceptor, oxygen (Créach et al., 2003). Tetrazolium dyes are reduced from a colourless complex to a brightly coloured, intracellular formazan precipitate by components of the electron transport system and/or a variety of dehydrogenase enzymes present in active bacterial cells (Joux and Lebaron, 2000).

In this PhD thesis a variant approach is applied, using the redox dye 5-cyano-2,3-ditolys tetrazolium chloride (CTC). This dye is reduced by bacteria to water-insoluble, red fluorescent formazan crystals and this red fluorescence can be detected flow cytometrically, indicating the degree of respiratory activity (Braux et al., 1997; Boulos et al., 1999; Joux and Lebaron, 2000).



2.4.2.6 Cellular energy

Adenosine triphosphate (ATP) is the key energy-containing molecule for most cell types and as such drives numerous energy-consuming reactions e.g. biosyntheses, mechanical motility, transport through membranes and regulatory networks (von Ballmoos et al., 2009). Indeed, ATP is a very important indicator of the metabolic state and health of cells, including bacteria (Aoki et al., 2009; von Ballmoos et al., 2009). In bacteria, ATP can be generated either through oxidative phosphorylation, by creating a proton motive force (PMF) that drives ATPsynthase, or through substrate level phosphorylation (e.g. glycolysis). Additionally, certain bacteria can accumulate energy-rich compounds like phosphoenolpyruvate, glycogen or triacylglycerides, which can be converted to ATP under starvation conditions (Hammes et al., 2011).

ATP-associated bioluminescence has been widely used as indicator of microbial activity, also in this PhD thesis. The principle of the ATP bioluminescence technique assay is based on an enzyme-substrate reaction. The luciferase-luciferin complex typically used these assays, converts the chemical energy associated with ATP into light. The luminescent signal is thus proportional to the ATP concentration, which is in turn directly proportional to the number of cells in culture (Chu et al., 2001; Lehtinen et al., 2004).

2.4.2.7 Presence of (intact) nucleic acids

Nucleic acids are large biopolymers essential for all forms of life. They are highly important for encoding, transmitting and expressing genetic information. The loss of intact nucleic acids will disable the cell to perform these most basic cellular processes (Hammes et al., 2011).

The correlation between cell viability and detection of DNA was earlier considered to be poor (Masters et al., 1994). However, a range of molecular targets have been successfully used in microbiological assays. Analysis based on the polymerase chain reaction (PCR) and DNA microarrays can detect bacterial DNA sequences, and identify and enumerate bacterial species (Ye et al., 2001; Keer and Birch et al., 2003). Quantitative PCR can be used to study bacterial viability in combination with ethidium monoazide (EMA). The latter is added to a bacterial sample and will only enter membrane-damaged cells, where it will covalently bind to the DNA and stain it. Only unstained DNA from viable cells will be amplified by PCR (Rudi et al., 2005).

Attention also turned to the use of messenger RNA (mRNA) as a marker of viability, as mRNA is a highly labile molecule with a very short half-life (seconds) and therefore should be a better indicator for viability than DNA. Ribosomal RNA (rRNA) can also be used as an indicator of viability (McKillip et al., 1999; Villarino et al., 2000) and has been found to be positively correlated with bacterial viability under certain circumstances (McKillip et al., 1999). Recently, the use of pre-rRNA has also been described (Cangelosi et al., 2010).



In this PhD thesis, the antibacterial potential of glycine and its *N*-methylated derivatives *N*-methylglycine (sarcosine), *N,N*-dimethylglycine (DMG) and *N,N,N*-trimethylglycine (betaine) is investigated, based on the assessment of complementary viability parameters.

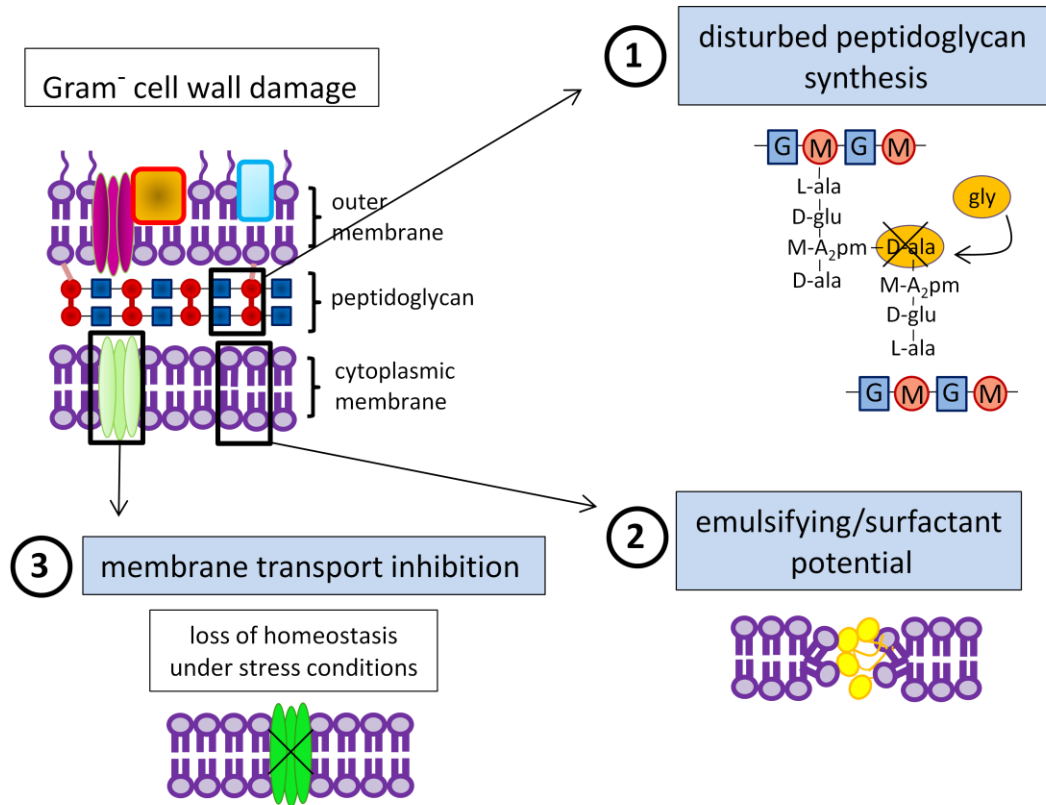
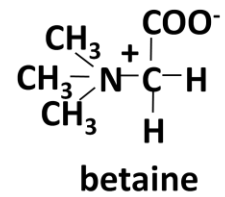
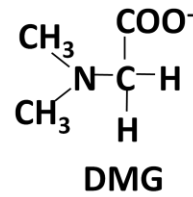
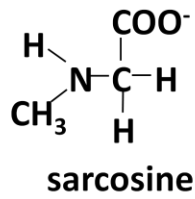
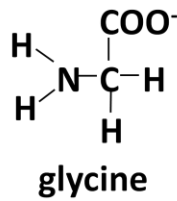


3 THE ANTIMICROBIAL POTENTIAL OF GLYCINE DERIVED COMPOUNDS

The antimicrobial effect of a molecule can be defined as the negative interaction between this active substance and specific targets in the microbial cell. These targets can be the cell wall, the cytoplasmic membrane, membranar enzymes, cytoplasmic components and genetic material (Kohanski et al., 2010). Although it is a common natural amino acid and not perceived as an antimicrobial, literature provides several indications that glycine and some of its derivatives could have an antimicrobial potential. Of relevance for this PhD thesis, Fig. 3 presents the clues leading to possible antibacterial characteristics of glycine and its *N*-methylated derivatives sarcosine, DMG and betaine.



Alkaline pH
pH 9.5



Physiological pH
pH 7.4

Change in ionization state

4 enhanced H⁺ consumption

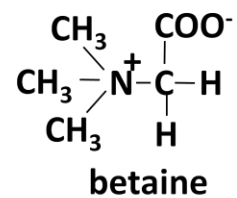
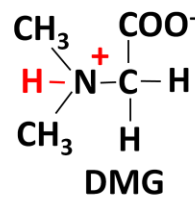
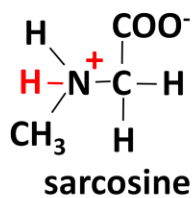
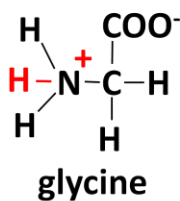


Figure 3: Indications for possible antibacterial characteristics of glycine and its *N*-methylated derivatives sarcosine, DMG and betaine. (1) glycine disturbs peptidoglycan synthesis. (2) emulsifying/surfactant potential described for the test compounds and their derivatives. (3) potential loss of bacterial homeostasis by inhibition of certain membrane transport systems. (4) the ionization state of glycine, sarcosine and DMG is dependent on the environmental pH



3.1 The influence of glycine on the bacterial cell wall

The Gram negative cell wall consists of an outer membrane, a peptidoglycan layer and a cytoplasmic membrane. For glycine, a mechanism of bacterial growth inhibition involving damage to the Gram negative cell wall has been described. Glycine disturbs bacterial cell wall synthesis, leading to increased cell wall permeability and potentially bacterial lysis. An excess of glycine inhibits the growth of various Gram positive, as well as Gram negative bacteria (Dempsey et al., 1973; Hammes et al., 1973; Ikura et al., 1986; Heaton et al., 1988; De Jonge et al., 1996; Hu et al., 2002; Minami et al., 2004; Li et al., 2010).

Glycine can replace the D- alanine residues on the *N*-acetylmuramic acid (MurNAc) subunits of the cell wall peptidoglycan. Replacement of D-alanine with glycine leads to the construction of defective peptidoglycan precursors. Two mechanisms generating a more loosely cross-linked peptidoglycan are proposed (Hammes et al., 1973). First, the accumulation of glycine-containing precursors may create an imbalance between the synthesis and the controlled enzymatic hydrolysis of peptidoglycan during growth. Second, the glycine-substituted MurNAc may be incorporated during peptidoglycan synthesis, causing a high percentage of uncross-linked muropeptides. Both mechanisms finally lead to a defective bacterial cell wall.

3.2 Potential emulsifying/surfactant characteristics

Amino acid-derivatives have been attributed membrane altering characteristics by several authors (Clapés and Infanté, 2002; Sánchez et al., 2007; Faustino et al., 2010). Moreover, amino acids themselves have an ampholytic nature, making them potentially interesting candidate emulsifiers/surfactants (Zajic and Panchal, 1976). Amino acid-based surfactants are characterized by good biocompatibility and low toxicity (Clapés and Infante, 2002).

Sarcosine, the monomethyl derivative of glycine is involved in a variety of biological processes as it is an intermediary metabolite in the choline, betaine and homocysteine metabolism. It is attributed various beneficial effects in human mental health care (Lane et al., 2006; Friesen et al., 2007).

DMG, the dimethyl-derivative of glycine is also an intermediary metabolite in the choline, betaine and homocysteine metabolism. It naturally occurs in low levels in cereal grains, seeds, beans and liver, but can also be formed by alkylation of glycine (Kalmar et al., 2010). It can act as a methyl donor (Friesen et al., 2007) and is attributed anti-oxidative properties (Hariganesh and Prathiba, 2000). Some studies even report improved athletic performances in both men and animals, improved immune responses and reduced blood lactate levels (Tonda and Hart, 1992; Greene et al., 1996).



Betaine, the trimethyl derivative of glycine, is also a naturally occurring compound and is widely distributed in many plants and animal tissues (Ratriyanto et al., 2009). It is used both as a feed additive in animal nutrition and as a food supplement for humans, because of its functions as a methyl group donor, compatible solute and its role in the homocysteine metabolism (Eklund et al., 2006b; Atkinson et al., 2008). It is known to improve feed efficiency, growth performance and carcass composition (Eklund et al., 2005; Matthews et al. 2001).

Besides the applications as food supplements, sarcosine-, DMG- and betaine-derived molecules are used as surfactants in industrial applications (Guan and Tung, 1998; Clapés and Infante, 2002). Enhanced emulsification of dietary fat by use of DMG, due to its surfactant properties, has also been suggested by Kalmar et al. (2011). If glycine and its *N*-methylated derivatives do indeed possess emulsifying characteristics, they could have a direct negative action on the bacterial cytoplasmic membrane. This could then lead to a loss of viability as a consequence of direct membrane damage.

3.3 Inhibition of membrane transporters

Glycine itself can be acquired from the cell surroundings by the *cycA* transporter (Saier, 2000). This is a broad substrate specific symporter which is also capable of importing serine and alanine. Its transcription levels are increased in *E. coli* under ammonia deficiency conditions (Hua et al., 2004), which is the case in our experimental setup.

The role of betaine as a so-called “compatible solute” is well described for *E. coli* and several other bacterial species (Culham et al., 2001; Ly et al., 2004; Tøndervik and Strøm, 2007). Compatible solutes can be defined as small organic molecules with specific characteristics (Gutierrez et al., 1995): (i) they are soluble to a high concentration and can be accumulated intracellular to very elevated levels without being toxic, (ii) they are often electrically neutral or Zwitterionic molecules, which need to be either actively transported across the cytoplasmic membrane, or be synthesized *de novo* by certain bacterial species.

Betaine is preferably transported into osmotically stressed *E. coli* via the membrane porters ProP and ProU (Csonka, 1989; Peddie et al., 2003; Wood, 2006; Wood, 2007). Interestingly, MacMillan et al. (1999) describe an inhibitory role (competitive inhibition) for DMG and sarcosine on the uptake of the osmolyte proline by the ProP osmoporter. This finding suggests that both glycine derivatives may have an effect on the *E. coli* membrane pumps required to maintain homeostasis under several stress conditions. Ammonia deficiency upregulates the transcription levels of the ProP and *amtB* gene (ammonium and methylammonium transport) (Hua et al., 2004). ProU also transports solutes such as betaines, proline, choline, DMG... from the extracellular environment to the intracellular bacterial cytoplasm. Its expression is induced both by hyperosmotic conditions and by alkaline pH (pH 8.2) (Smirnova and Oktyabrsky, 1995). This provides a strong indication that ProU plays a role in pH



stress as well as osmotic stress. Moreover, the response of *E. coli* to betaine at this alkaline pH was similar to that observed in a high osmolarity medium (Smirnova and Oktyabrsky, 1995). Additionally, there are indications of overlapping mechanisms and roles for osmolytes in osmotic homeostasis and pH homeostasis (Wang et al., 2007; Kitko et al., 2010). The expression of the ProU gene in *E. coli* encoding for this osmotically inducible transport system is influenced by alkaline changes in cytoplasmic pH (Smirnova and Oktyabrsky, 1995), providing a strong argument for a protective role of betaine under alkaline stress. The overlap between osmotic and pH stress compensation mechanisms could help in identifying possible antibacterial effects of the compounds at hand.

Alkali conditions also induce differential regulation of many open reading frames (ORFs) coding for ABC transporters. These ABC transporters play an important role in various cellular physiological processes, including uptake of oligopeptides and other solutes under alkaline conditions (Padan et al., 2005). Our compounds can be regarded as solutes and could be taken up by such transporters.

3.4 The importance of the ionization state

Many antimicrobial molecules are weak acids or weak bases. This implies that they contain at least one functional group that can reversibly donate or accept a proton to form a negatively charged anion or a positively charged cation respectively. The reversibility of this reaction leads to a proton scavenging or releasing effect of these compounds when environmental pH conditions are varied.

A well-known example hereof is that of the organic acids. Their antibacterial effect is partially based on the release of protons as they enter the cytoplasm of a microorganism (Van Immerseel et al., 2006; Lu et al., 2011). This intracellular deprotonation has severe consequences: lowering of the cytoplasmic pH leading to a disturbance of pH homeostasis and anion accumulation leading to osmotic stress (Booth 1985, Roe et al., 2002). This forces the cell to spend more energy to extrude protons for pH homeostasis. This consumption of energy drains the cell of its ATP and will possibly lead to cell death (Hosein et al., 2011).

Another example of the influence of changes in intracellular proton concentrations on bacterial viability is described by Yohannes et al. (2005). Of relevance for this PhD thesis, these authors describe an antibacterial effect of polyamines under alkaline conditions. As the underlying mechanisms they suggest that the amine group of these molecules is protonated when the polyamines pass from an alkaline environment to the more acidic cytoplasm. This leads to a consumption of cytoplasmic protons, causing an intracellular alkalinization. The cell now has to pump in protons for its pH homeostasis. This proton pumping action will, as described above, drain the cell from its energy source, possibly leading to a loss in viability.

As glycine and its *N*-methylated derivatives sarcosine and DMG (but not betaine) also have a primary, secondary or tertiary amine group (in contrast to the quaternary ammonium group of betaine) that can

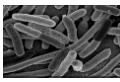


function as a proton acceptor. This implies that glycine, sarcosine and DMG could also have the potential to amplify pH stress by scavenging protons within the cytoplasm, causing a decreased bacterial viability.



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Scientific Aims



Literature suggests a potential antibacterial effect of glycine and its *N*-methylated derivatives sarcosine, DMG and betaine. A working mechanism is yet to be identified. Viability analysis can elucidate the effects of these compounds on specific cellular compartments.

The aims of this PhD thesis were:

- 1) to quantify the effect of glycine and its *N*-methylated derivatives sarcosine, DMG and betaine on the viability of *E. coli* under different pH conditions, by determination of traditional culture-based and complementary non-culture based viability assessment methods
- 2) to explore the working mechanism of this antibacterial effect based on in-depth viability analysis, targeting different cellular compartments
- 3) to confirm and extend this proposed working mechanism by assessing the antibacterial effects of a parallel series of amine-based compounds.

The envisaged benefits of the research are two-fold:

- 1) Knowledge on the mode of action of glycine and its *N*-methylated derivatives can help to identify the antibacterial potential of other interesting compounds
- 2) Validation of the concept of viability loss for *E. coli* as a model pathogen provides opportunities to verify this mode of action in other microorganisms

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Experimental Studies

Chapter 1

Glycine and its *N*-methylated analogues cause pH-dependent membrane damage to enterotoxigenic *Escherichia coli*

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ABSTRACT

The current study first investigates the emulsifying potential of glycine and its *N*-methylated derivatives *N*-methylglycine (sarcosine), *N,N*-dimethylglycine (DMG) and *N,N,N*-trimethylglycine (betaine) under varying pH conditions. Subsequently, the effect of these test compounds on the membrane integrity of enterotoxigenic *Escherichia coli* (ETEC, *E. coli*) was evaluated. Oil in water emulsions containing each compound show that DMG is a more potent enhancer of emulsification than glycine, sarcosine and betaine under the conditions tested. Flow cytometry was used to investigate whether the emulsifying potential is associated with an effect on *E. coli* membrane integrity. The bacteria were exposed to each of the test compounds under varying pH conditions and membrane integrity was assessed using the LIVE/DEAD BacLight kit. Results show a membrane deteriorating effect caused by glycine, sarcosine and DMG, but not by betaine. This effect is pH- and time-dependent and has an apparent threshold at pH 9.0. Conventional plate counts confirmed concomitant changes in culturability of the membrane-comprised bacteria.



INTRODUCTION

N,N,N-trimethylglycine (betaine) is used as a feed additive in animal nutrition because of its functions as a methyl group donor and compatible solute (Eklund et al., 2006b). It is known to improve feed efficiency, growth performance and carcass composition (Eklund et al., 2005; Matthews et al. 2001). Furthermore, crude fat digestibility is improved by dietary betaine supplementation (Eklund et al., 2006a). Much less information is available on *N,N*-dimethylglycine (DMG) as a dietary supplement in livestock production. However, recent data on the use of DMG as a feed additive also show beneficial effects on performance in broilers (Kalmar et al., 2010b, 2011) and on nutrient digestibility in broilers (Kalmar et al., 2010a) and pigs (Cools et al., 2010). The effects on nutrient digestibility are suggested to result from an emulsifying action at the gut level. Both DMG and betaine derived molecules are used as surfactants in industrial applications (Guan and Tung, 1998; Clapés and Infante, 2002). Enhanced emulsification of dietary fat by use of additives with surfactant properties is suggested to facilitate liberation of non-fat nutrients from a fatty insulation, rendering them sooner available for enzymatic digestion and absorption through which digestibility is improved (Kalmar et al., 2011). Therefore, the first objective of this study was to investigate the emulsifying potential of glycine and its *N*-methylated analogues sarcosine, DMG and betaine.

Recent data further suggest that betaine might modify the microbial composition in the small intestine of pigs. Dietary supplementation with betaine improves fibre digestibility in weaned piglets (Eklund et al., 2006a/b; Ratriyanto et al., 2010). Metzler-Zebeli et al. (2009) report that betaine supplementation of pig feed tends to stimulate intestinal bacterial growth, *i.e.* both of beneficial microbiota and of enterobacteria such as pathogenic *E. coli*. However, further information on the impact of betaine and other *N*-methylated analogues of glycine on the intestinal microbial populations in pigs is lacking.

Enterotoxigenic *Escherichia coli* (ETEC) is worldwide the most common bacterial cause of diarrhoea in humans and various animal species (Walker et al., 2007). It is also an important cause of postweaning diarrhoea in piglets. This type of diarrhoea is responsible for considerable economic losses due to a decreased growth rate and an increased mortality (Fairbrother et al., 2005). The bacterial cytoplasmic membrane is a phospholipid bilayer (Nelson et al., 2009) that acts as a permeability barrier which is sensitive to emulsifying molecules. The second objective of this study was therefore to investigate whether the emulsifying potential assigned to betaine and DMG affect *E. coli* membrane integrity.

Along its path in the digestive tract the *E. coli* are exposed to a wide range of pH values. As they pass through the pylorus and enter the upper small intestine, they encounter alkaline pancreatic secretions, resulting in a highly alkaline environment (Stancik et al., 2002). Recently, the existence of alkaline surface microclimates in the small intestine was described, which are related to the working mechanism of alkaline phosphatase (AP) (Mizumori et al., 2009). The latter enzyme is an alkaline



chemosensor regulating the small intestinal surface pH and is predominantly found on the apical surface of the differentiated enterocyte in post-weaned animals (Lallès, 2010). The optimal pH of the alkaline phosphatase activity in pigs is 10.5 (Fan et al., 1999). Therefore, even though the chime-pH is generally below pH 7.0 (Snoeck et al., 2004), the investigated pH-range in the current study extends to a much more alkaline pH value associated with alkaline phosphatase activity.

Changes in environmental pH not only have an influence on bacterial homeostasis mechanisms (Padan et al., 2005), but also affect the emulsifying capacity of various surfactants (Abouseoud et al., 2010). The third objective of this in vitro study was therefore to investigate whether environmental pH changes influence either the emulsifying potential and/or the effect on *E. coli* membrane integrity of glycine and its *N*-methylated analogues.

MATERIALS AND METHODS

Test compounds

Glycine ($\geq 99\%$ purity), sarcosine ($\geq 99\%$ purity) and betaine ($\geq 99\%$ purity) were obtained from Sigma Aldrich, DMG ($\geq 97\%$ purity) was obtained from Taminco (Taminco N.V., Ghent, Belgium). All compounds were stored to manufacturers' guidelines until use. Compound characteristics are summarized in Table 1.

	structure	pK _a amine group	pK _a carboxylic acid group
glycine		9.78	2.35
sarcosine		10.12	2.21
DMG		10.01	1.87
betaine		-	1.84

Table 1: Chemical structures and pK_a values of glycine and its *N*-methylated analogues



Emulsifying potential

Emulsifying properties were first measured by the method described by Wu et al. (1998). Briefly, pure corn oil (2 ml) and 6 ml of 1% test compound solutions in distilled water (pH ranging from 6.5 to 10.0) were homogenized using an Ultraturax at the highest setting for 1 min. Fifty-microliter portions of the emulsion were pipetted from the bottom of the container at 0 and 5 min after homogenization. Each portion was diluted with 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. After vortexing, the absorbance of the dilutions was measured spectrophotometrically at 500 nm (Genesys 10UV, Spectronic Unicam).

The emulsion stability index (ESI) was calculated using the following equation: $ESI (min) = A_0 \times (\Delta T / \Delta A)$, where A_0 and A_5 are the absorbances of the diluted oil in water emulsions at 0 and 5 minutes after homogenization, respectively, $\Delta T = 5$ min and $\Delta A = A_0 - A_5$. The emulsifying activity index (EAI) was calculated using the following equation: $EAI (m^2/g) = 2T * ((A_0 * DF)/(C * \phi * 10000))$, where $T=2.303$; DF =dilution factor; C = weight of compound/unit volume (g/mL) of aqueous phase before emulsion formation; ϕ = oil volume fraction of the emulsion.

Secondly, the red blood cell lysis capacity was measured using an adapted the protocol from the haemolysis assay described by Pape et al. (1999). Erythrocytes were obtained from the blood of healthy pigs collected in heparin-coated tubes. They were washed 3 times in sterile phosphate buffered saline (PBS) and then resuspended with PBS to the original volume. A 50 mM solution of each test compound was made in sterile PBS. The pH of these solutions was adjusted to pH 6.5, 9.0, 9.5 or 10.0 by either HCl or NaOH addition. The pH-adjusted sterile PBS solution was used as negative control, while distilled water served as positive control. The 10 μ l aliquots of erythrocyte suspension were exposed to each of these solutions, in a total volume of 200 μ l. Following incubation at 37°C for 3 hours (h) and centrifugation at 1300 g, the absorbance of the supernatant was measured spectrophotometrically at 550 nm. All data are the result of triplicate experiments.

The percentage of haemolysis was calculated for each sample by dividing its absorbance by the positive control absorbance (complete haemolysis) and multiplying this value by 100 (Gould et al., 2000).

Bacterial strain and culture conditions

The hemolytic ETEC strain GIS26, serotype 0149:F4ac, positive for heat labile (LT) and heat stabile (STa and STb) enterotoxins (Van den Broeck et al., 1999), was grown overnight at 37°C in brain heart infusion medium (Oxoid Limited, Hampshire, United Kingdom) to stationary phase. Bacteria were collected by centrifugation (10'000 x g, 2 minutes, room temperature) of 1 ml of the bacterial culture containing approximately 10^9 bacteria and then resuspended in each of the appropriate test compound solutions in 0.85% NaCl. The initial bacterial concentration was determined by colony plate counts



Minimum inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) for the test compounds was performed using the agar dilution assay (CLSI, 2008), using Mueller-Hinton agar. Plates were incubated at 35°C (+/-2°C) for 16-20 hours (h) in an aerobic atmosphere.

Flow cytometric assessment of bacterial membrane integrity

A 50 mM solution of each test compound was made in sterile saline. The pH of these solutions was adjusted to 6.5, 8.5, 9.0, 9.5 or 10.0 by HCl or NaOH addition. Overnight grown *E. coli* were dispersed in 1 ml of each test solution to a concentration of $\sim 10^9$ bacteria/ml. As a control, pH-adjusted sterile saline was used. The bacterial suspensions were incubated shaking for 1, 3, 6 and 20h at 37°C. After incubation, bacterial cells were collected by centrifugation (10'000 x g, 2 minutes, RT), washed with sterile saline and finally resuspended in 1 ml of sterile saline.

Membrane integrity was assessed using the LIVE/DEAD BacLight™ kit (Molecular Probes Eugene, OR, USA) as described by the manufacturer. This bacterial viability kit is widely used in flow cytometry and consists of two nucleic acid stains. Green fluorescent SYTO9 is cell-permeable and can freely enter all *E. coli*, either live or dead. In contrast, red fluorescent propidium iodide (PI) can only enter membrane-compromised cells. Green fluorescence was collected in the FL1 channel (530 ± 15 nm) and red fluorescence in the FL3 channel (>670 nm). All parameters were collected as logarithmic signals. To acquire the correct settings of voltages and compensations different proportions of live and dead bacteria were mixed to obtain cell suspensions containing various ratios. Populations were first single stained (SYTO9 or PI) and subsequently double stained (either SYTO9 and PI mixture).

In our set-up 10 µl of the treated bacterial cell suspension was added to 987 µl of sterile saline. These samples were immediately stained with 3 µl of a mixture of SYTO9 (5 µM final concentration) and PI (30 µM final concentration) and incubated for 15 minutes in the dark at RT. Flow cytometric measurements were performed immediately thereafter, using a FACSCanto flow cytometer (Becton, Dickinson and Company, Erembodegem, Belgium). Minimally 10 000 events were recorded. All data on the percentages of live (L), intermediate (I) and dead (D) bacteria (all together approaching 100%) were acquired and processed using FACS Diva software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Plate counts

The number of colony forming units (CFU) per ml was assessed by conventional plate count, which is based on CFU values obtained from a 10-fold serial dilution of each sample plated on Tryptone Soy Agar (Oxoid Limited, Hampshire, United Kingdom) and incubated overnight at 37°C. These plate counts determine the number of culturable bacteria in each sample.



Statistical Analysis

ESI-values were normally distributed and homoscedastic. Effects of the test compounds and of pH revealed significant interaction in a two-factor ANOVA. For the compound effect at different pH values, one-way ANOVA and post-hoc comparisons with a Bonferroni adjustment were applied. Correlation and stepwise linear regression were further used to analyze the influence of pH on ESI, where effects coding accounted for the compound present.

Flow cytometric data of the percentages of live, intermediate and dead bacteria were arcsine-transformed to obtain normal distributions; CFU values obtained by plate counts were logarithmically transformed. In order to compare the effects of the compounds after 20h incubation with the control condition at each pH, one-way ANOVA was performed and followed by Dunnett multiple comparisons to the control condition. When however the variances were not homogenous (Levene's test), Welch' robust variation of ANOVA was used and followed by Dunnett's T3 multiple comparisons. A simultaneous piecewise regression (broken stick) was used to describe the influence of pH on the percentage of live bacteria, where reference coding versus the control condition accounted for the presence of a compound.

Statistical significance: $p < 0.05$.

RESULTS

Emulsifying potential

Fig. 1 presents the emulsion stability index (ESI) of sarcosine, DMG and betaine, compared to the non-methylated amino acid glycine under varying pH conditions. ESI data are the result of triplicate experiments, mean values $\pm$ SD are presented in Table 2. EAI data are presented in Fig. 2.

Results show that DMG has the strongest ESI ($p < 0.0005$) compared to glycine, sarcosine and betaine at all pH values tested. The effect of pH on ESI differs between glycine, sarcosine and betaine. At pH 6.5; 8.5 and 9.5 there is no significant difference between these 3 test compounds. At pH 9.0 glycine has a significantly higher ESI than betaine, and at pH 10.0 sarcosine has a significantly higher ESI than betaine. However, over the whole pH range there is no significant difference between glycine, sarcosine and betaine. These results suggest that there is no influence of methylation degree on the ESI.

There is a significant (positive) regression of ESI on pH, with ESI increasing by 0.42 ± 0.19 ($p = 0.045$) and 0.57 ± 0.13 ($p = 0.001$) minutes per pH-unit for glycine and sarcosine, respectively, but not for DMG ($p = 0.88$) and betaine ($p = 0.26$).

No haemolytic effect of glycine or its 3 *N*-methylated analogues was observed at pH 6.5, 9.0, 9.5 or 10.0. This strongly indicates that there is no surfactant-like effect on the erythrocyte cytoplasmic



membrane under the conditions applied. The absence of this haemolytic effect is also illustrated in Table 3.

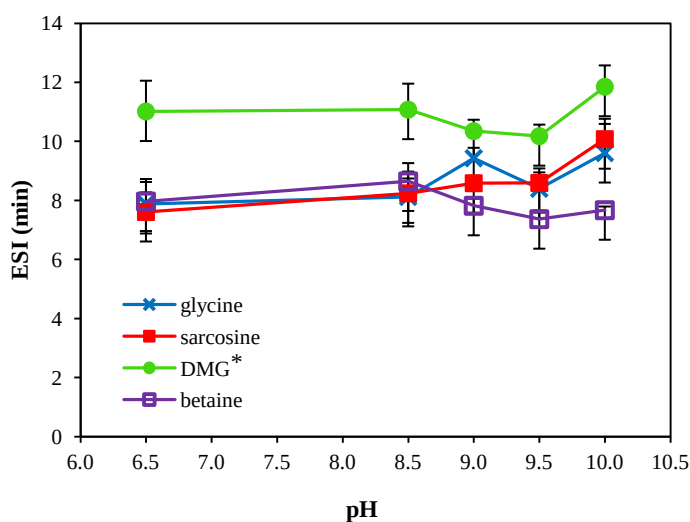


Figure 1: Emulsion stability index (ESI) of glycine, sarcosine, DMG and betaine. Data are expressed as means $\pm$ SD of triplicate experiments. DMG had the strongest ESI ($p < 0.0005$) compared to glycine, sarcosine and betaine at all pH values tested. There is a significant (positive) regression of ESI on pH, with ESI increasing by 0.42 ± 0.19 ($p = 0.045$) and 0.57 ± 0.13 ($p = 0.001$) minutes per pH-unit for glycine and sarcosine respectively, but not for DMG ($p = 0.88$) and betaine ($p = 0.26$)

treatment	pH				
	6.5	8.5	9.0	9.5	10.0
glycine	7.88 $\pm$ 0.85	8.12 $\pm$ 0.86	9.43 $\pm$ 0.35	8.40 $\pm$ 0.69	9.61 $\pm$ 1.16
sarcosine	7.61 $\pm$ 0.67	8.24 $\pm$ 0.50	8.58 $\pm$ 0.25	8.59 $\pm$ 0.36	10.08 $\pm$ 0.52
DMG	11.01 $\pm$ 1.04	11.07 $\pm$ 0.88	10.35 $\pm$ 0.39	10.18 $\pm$ 0.39	11.85 $\pm$ 0.72
betaine	7.96 $\pm$ 0.66	8.65 $\pm$ 0.62	7.82 $\pm$ 0.24	7.37 $\pm$ 0.30	7.67 $\pm$ 0.13

Values represent mean values $\pm$ standard deviation of at least triplicate experiments

Table 2: Emulsion stability index (ESI) of glycine, sarcosine, DMG and betaine at different pH values

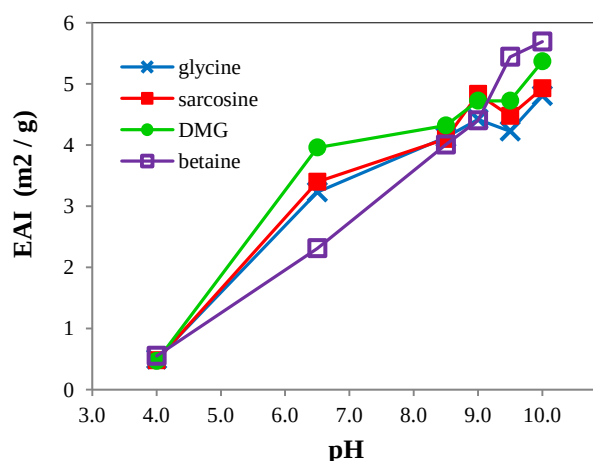


Figure 2: Emulsifying activity index (EAI) of glycine, sarcosine, DMG and betaine at different pH values

Hemolysis	pH				Positive control (100% hemolysis)
Compound (50 mM)	6.5	9.0	9.5	10.0	
Glycine					
Sarcosine					
DMG					
Betaine					
Control					

Table 3: Representative images of the microplate hemolysis assay. Erythrocyte suspensions (10 μ l) were exposed to the test compounds solutions (50 mM) in a total volume of 200 μ l. Following incubation, the absorbance of the supernatant was measured at 550 nm in a spectrophotometer. The percentage of hemolysis was calculated for each sample by dividing the sample absorbance by the positive control absorbance (100% hemolysis) multiplied by 100 (Gould et al., 2000)



Minimum Inhibitory Concentration (MIC) determination

Concentrations ranging from 1 mM up to 100 mM of each test compound were investigated for antibacterial potential against *E. coli*. None of the tested compounds and concentrations inhibited visible bacterial growth (data not shown). Therefore, the MIC values were above 100 mM for the 4 compounds tested.

Flow cytometric measurement of membrane permeability

Membrane integrity analysis with SYTO9/PI revealed a unique fluorescence pattern for the bacterial cell population which is directly related to the degree of membrane damage. As demonstrated in Fig. 3, three bacterial subpopulations could be identified: live (L), intermediate (I) and dead (D). With increasing membrane permeability the bacterial population shifts from high a SYTO9/low PI fluorescence intensity to a state with an even further increased SYTO9 fluorescence intensity, and then to a state of high PI/low SYTO9 fluorescence intensity.

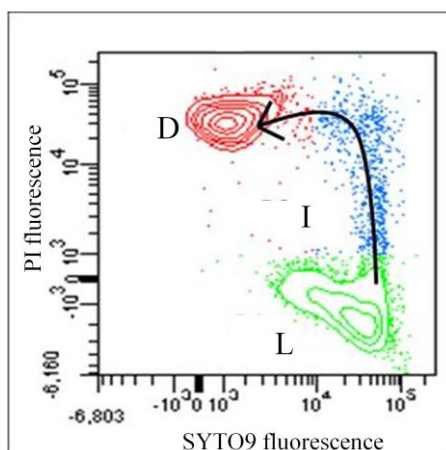


Figure 3: Flow cytometric SYTO9/PI dot plot presenting a population of *E. coli* at different stages of membrane damage. The arrow specifies changes in the position of various bacterial subpopulations moving from live (L) over intermediate (I), to dead (D)

Percentages of live, intermediate and dead bacteria are the result of triplicate experiments and all data are presented in the table 4. At pH 6.5 and 8.5 the membrane of the bacterial cells was not affected by any of the test compounds. Results show a clear pH-dependent increase in membrane permeability when exposed to glycine, sarcosine and DMG, but not betaine, at pH 9.0 to 10.0. Illustrative flow cytometric dot plots of *E. coli* samples incubated with DMG for 6h at the various pH values are presented in Fig. 4, while Fig. 5 presents the live and dead bacterial subpopulations after 20h of incubation with each of the 4 test compounds and saline as a control, at a pH ranging from pH 6.5 to pH 10.0.

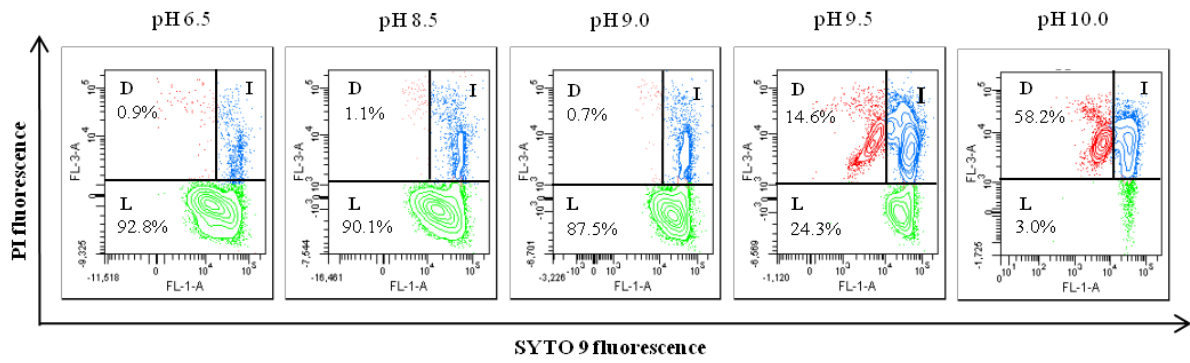


Figure 4: Flow cytometric SYTO9(FL1)/PI(FL3) dot plots presenting pH dependent *E. coli* membrane damage after incubation with 50 mM DMG for 6h at a pH ranging from 6.5 to 10.0. The L region corresponds to live cells with intact membranes. The I region corresponds to bacteria in an intermediate injured state with comprised membranes. The D region corresponds to dead cells. Percentages are the mean of triplicate experiments

pH	treatment 50 mM	Incubation time											
		1h			3h			6h			20h		
		L	I	D	L	I	D	L	I	D	L	I	D
6.5	glycine	94.0±1.0	5.4±1.1	0.6±0.1	94.9±0.6	4.7±0.7	0.4±0.1	94.1±1.7	5.0±0.8	1.1±1.1	85.1±3.6	12.1±2.9	2.9±1.5
	sarcosine	93.7±1.1	5.6±0.9	0.8±0.2	93.7±0.7	5.8±0.6	0.6±0.2	84.6±3.6	14.8±3.4	0.9±0.3	83.2±2.8	13.8±2.3	3.6±0.4
	DMG	92.1±2.4	7.2±2.2	0.8±0.2	92.5±1.3	7.0±1.1	0.6±0.3	92.8±1.1	6.4±0.8	0.9±0.4	80.0±1.1	18.1±0.8	2.1±0.8
	betaine	91.7±1.8	7.7±1.7	0.7±0.4	91.3±1.2	8.5±1.2	0.4±0.2	89.5±2.3	9.7±2.4	0.8±0.1	82.2±2.5	14.3±2.1	3.7±0.6
	control	93.7±1.8	5.8±1.4	0.6±0.3	93.8±0.6	5.8±0.6	0.5±0.0	89.0±1.3	10.4±1.2	0.8±0.2	72.7±8.3	22.3±6.9	9.4±7.4
8.5	glycine	93.9±1.9	5.5±1.5	0.7±0.4	91.9±1.8	7.6±1.6	0.7±0.3	90.7±0.6	8.8±0.4	0.7±0.2	68.4±1.6	27.3±1.8	3.0±2.1
	sarcosine	93.9±1.7	5.4±1.6	0.8±0.2	93.9±1.0	5.5±0.7	0.7±0.3	80.2±4.7	19.2±4.6	1.1±0.2	78.4±2.2	18.5±2.9	3.8±0.4
	DMG	90.2±0.7	9.0±0.8	0.9±0.3	92.9±1.5	6.5±1.3	0.7±0.3	90.1±1.6	8.9±1.6	1.1±0.2	61.5±8.6	36.5±8.6	2.5±0.4
	betaine	92.1±1.9	7.1±1.9	0.9±0.1	92.2±0.3	7.1±0.4	0.8±0.2	83.9±1.2	15.1±0.8	1.3±0.4	66.0±5.5	29.7±9.0	3.9±1.4
	control	93.4±1.5	6.0±1.4	0.7±0.1	93.5±1.7	5.9±1.3	0.6±0.4	89.1±1.1	10.2±1.3	0.8±0.3	62.4±3.1	35.6±3.7	2.7±0.8
9.0	glycine	95.4±1.7	4.2±1.6	0.5±0.2	93.3±0.8	6.3±0.5	0.6±0.3	80.6±1.6	18.3±1.7	1.2±0.2	64.7±4.2	24.5±3.8	11.2±3.1
	sarcosine	95.6±0.3	4.0±0.3	0.6±0.1	95.2±0.6	4.5±0.7	0.4±0.1	76.9±1.8	22.8±1.5	0.6±0.2	77.5±5.9	11.8±2.8	11.5±3.5
	DMG	93.9±1.9	5.0±0.6	0.5±0.3	94.5±0.8	5.1±0.6	0.5±0.2	87.5±1.9	11.9±1.8	0.7±0.3	66.0±6.7	23.3±4.1	11.2±7.8
	betaine	92.2±0.7	7.4±0.9	0.6±0.2	95.5±0.6	4.1±0.5	0.5±0.1	86.6±1.4	13.2±1.2	0.4±0.3	75.5±1.6	20.0±1.8	4.6±0.3
	control	94.5±1.1	4.9±1.1	0.7±0.2	95.7±0.5	3.8±0.5	0.5±0.1	92.8±1.6	6.6±1.5	0.7±0.2	70.7±6.3	27.1±5.9	2.5±0.7
9.5	glycine	93.5±0.7	6.1±0.9	0.5±0.2	74.8±2.5	24.8±2.3	0.7±0.3	22.1±1.5	48.5±1.4	29.7±2.9	28.4±7.7	14.9±0.6	56.9±7.7
	sarcosine	94.0±0.4	5.2±0.3	0.8±0.3	88.8±2.6	10.6±2.5	0.7±0.2	49.2±4.0	49.2±3.5	2.0±0.5	56.6±2.8	16.2±2.9	27.0±2.7
	DMG	94.1±1.1	5.2±0.9	0.8±0.2	87.7±2.0	11.4±1.9	1.0±0.2	24.3±3.8	61.4±3.1	14.6±6.9	24.3±2.1	21.7±1.1	53.8±1.6
	betaine	92.0±1.0	7.2±1.0	0.9±0.2	91.4±1.6	8.0±1.7	0.8±0.1	84.4±2.4	14.8±2.3	0.6±0.3	74.4±1.2	20.0±0.8	5.8±0.2
	control	93.5±1.9	5.9±1.9	0.7±0.2	93.0±0.8	6.3±0.8	0.8±0.1	90.9±0.7	8.6±0.8	0.7±0.1	70.0±3.1	26.8±2.3	3.5±1.2
10.0	glycine	72.6±1.9	27.0±2.3	0.7±0.3	4.6±0.6	79.8±1.1	15.7±1.5	2.9±0.4	33.0±0.5	64.2±1.3	0.8±1.0	5.9±0.4	93.7±1.0
	sarcosine	87.2±1.4	12.2±1.1	0.8±0.3	14.6±3.5	76.8±3.1	8.9±1.3	16.7±3.5	27.8±1.0	56.0±2.5	0.8±0.3	16.1±1.0	83.5±1.5
	DMG	67.7±5.1	31.2±5.5	0.6±0.1	1.1±0.1	89.0±1.1	10.4±1.7	3.0±1.4	38.9±2.4	58.2±3.5	0.2±0.1	21.7±0.8	79.5±0.5
	betaine	89.0±2.6	10.6±2.6	0.6±0.3	92.9±0.6	6.9±0.6	0.5±0.2	88.2±1.1	10.7±1.3	1.3±0.6	78.8±3.2	16.6±1.7	4.8±1.9
	control	92.9±0.9	6.6±0.9	0.7±0.1	93.5±1.9	6.1±1.8	0.5±0.3	87.2±1.5	11.6±1.4	1.4±0.2	70.3±7.7	24.2±5.1	5.8±2.6

Values represent mean values ± standard deviation of at least triplicate experiments.

L = live, I = intermediate, D = dead

Table 4: Summary of flow cytometric data on *E. coli* membrane integrity at different time points following incubation with saline (control), glycine, sarcosine, DMG and betaine at different pH values (expressed as mean % of total bacterial population incubated)



A significant increase in the percentage of dead bacteria was observed in the presence of glycine, sarcosine and DMG at pH 9.0 ($p<0.05$) and even more so at pH 9.5 and 10.0 ($p<0.01$). When exposed to the latter 3 compounds, the percentage of live bacteria rapidly decreased after 3h to 6h when incubated at pH 9.5 and after only 1h to 3h when incubated at pH 10.0 (time course data not shown). Table 5 presents the live, intermediate and dead bacterial subpopulations after a 20h incubation at pH 10.0. For glycine, sarcosine and DMG the intermediate subpopulation is located closer to the dead subpopulation on the flow cytometric dot plots, compared to betaine and the control sample where the intermediate subpopulation is situated closer to the live subpopulation. In these samples that were incubated for 20h at pH 10.0 the percentage of intermediates was significantly ($p<0.05$) lower for the bacteria incubated with betaine, in comparison with the saline-incubated control sample.

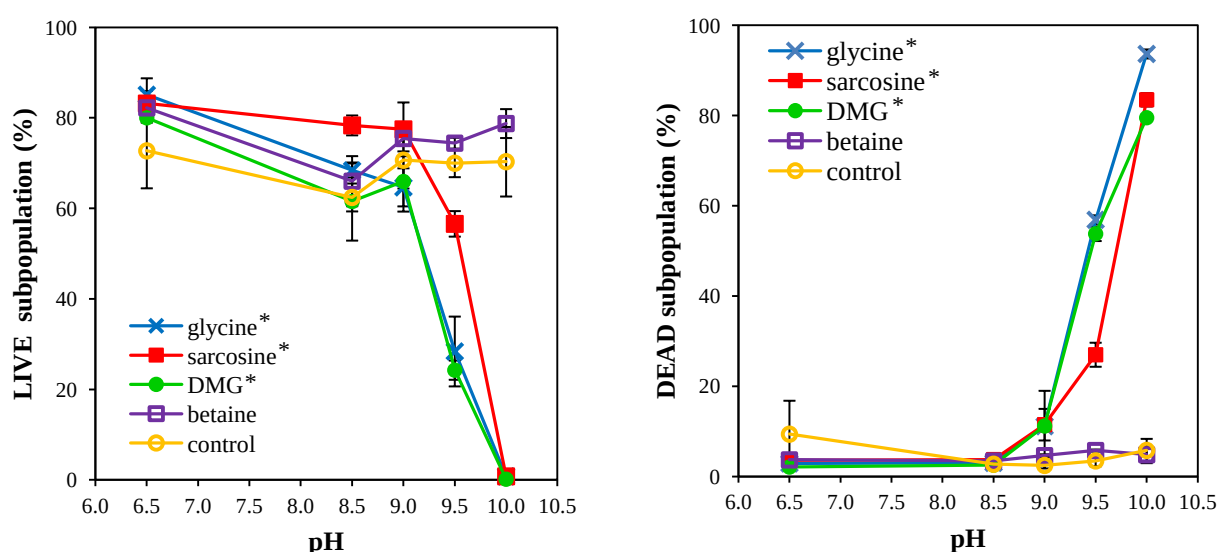


Figure 5: Percentage of live (L) and dead (D) *E. coli* subpopulations after 20h of incubation with glycine, sarcosine, DMG, betaine and sterile saline (control) at a pH ranging from 6.5 to 10.0. Data are expressed as means $\pm$ SD of triplicate experiments. There is a significant increase in percentage of dead bacteria in the presence of glycine, sarcosine and DMG at pH 9.0 ($p<0.05$) and higher ($p<0.01$)

	Live			Intermediate			Dead		
glycine	0.8 $\pm$ 0.95	*		5.9 $\pm$ 0.40	*		93.7 $\pm$ 1.01	*	
sarcosine	0.8 $\pm$ 0.26	*		16.1 $\pm$ 1.04	*		83.5 $\pm$ 1.48	*	
DMG	0.2 $\pm$ 0.12	*		21.7 $\pm$ 0.81			79.5 $\pm$ 0.49	*	
betaine	78.8 $\pm$ 3.20			16.6 $\pm$ 1.65	*		4.8 $\pm$ 1.86		
control	70.3 $\pm$ 7.68			24.2 $\pm$ 5.12			5.8 $\pm$ 2.56		

* $p<0.05$ (versus control)

Table 5: Percentage (mean $\pm$ SD) of live (L), intermediate (I) and dead (D) *E. coli* subpopulations after 20h of incubation with glycine, sarcosine, DMG, betaine and sterile saline (control) at pH 10.0



Plate counts

All data are the result of triplicate experiments, mean values $\pm$ SD are presented in Table 6. At pH 6.5 and 8.5 there was no significant difference between the plate counts of the *E. coli* samples incubated with saline, glycine, sarcosine, DMG or betaine. When incubated in more alkaline conditions, there was a pH- and time-dependent decrease in the number of culturable *E. coli* compared to saline when exposed to glycine, sarcosine and DMG, but not to betaine. The CFU counts of *E. coli* populations incubated with the different test compounds for 20h at pH 6.5 to 10.0 are presented in Fig. 6. Interestingly, differences in colony size in *E. coli* samples incubated with alkaline solutions of glycine, sarcosine and DMG were observed. Samples of bacteria incubated with the latter 3 test compounds formed smaller colonies as well as normal sized colonies. In contrast, there was no significant difference between the betaine and the saline incubated *E. coli* sample and no irregularly sized colonies were present at none of all the conditions tested.

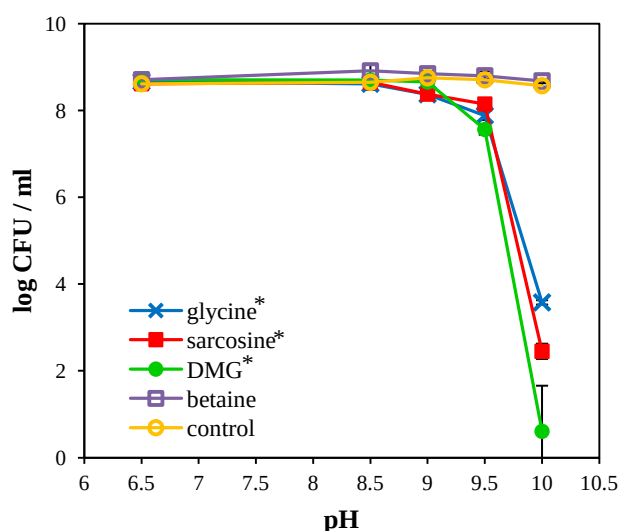


Figure 6: Culturability of *E. coli* after exposure to 50 mM of glycine, sarcosine, DMG, betaine and sterile saline (control) for 20h at a pH ranging from 6.5 to 10.0. There is a significant decrease in CFU/ml when exposed to glycine, sarcosine and DMG at pH 10.0 ($p < 0.01$)



pH	treatment 50 mM	population after indicated incubation time (log 10 CFU / ml)			
		1h	3h	6h	20h
6.5	glycine	8.80 ± 0.01	8.89 ± 0.03	8.91 ± 0.01	8.66 ± 0.07
	sarcosine	8.85 ± 0.05	8.86 ± 0.04	8.85 ± 0.02	8.61 ± 0.07
	DMG	8.76 ± 0.05	8.86 ± 0.01	8.85 ± 0.05	8.71 ± 0.10
	betaine	8.82 ± 0.03	8.87 ± 0.05	8.86 ± 0.03	8.71 ± 0.06
	control	8.82 ± 0.03	8.88 ± 0.01	8.87 ± 0.05	8.61 ± 0.13
8.5	glycine	8.81 ± 0.01	8.82 ± 0.04	8.86 ± 0.05	8.61 ± 0.14
	sarcosine	8.80 ± 0.02	8.91 ± 0.03	8.86 ± 0.04	8.65 ± 0.10
	DMG	8.84 ± 0.02	8.93 ± 0.03	8.86 ± 0.01	8.70 ± 0.10
	betaine	8.81 ± 0.03	8.91 ± 0.05	8.89 ± 0.06	8.91 ± 0.14
	control	8.87 ± 0.03	8.92 ± 0.01	8.93 ± 0.01	8.65 ± 0.16
9.0	glycine	8.84 ± 0.07	8.86 ± 0.08	8.82 ± 0.06	8.37 ± 0.11
	sarcosine	8.83 ± 0.08	8.85 ± 0.05	8.89 ± 0.02	8.37 ± 0.05
	DMG	8.83 ± 0.07	8.86 ± 0.10	8.90 ± 0.06	8.65 ± 0.15
	betaine	8.85 ± 0.06	8.85 ± 0.05	8.89 ± 0.01	8.84 ± 0.07
	control	8.80 ± 0.09	8.81 ± 0.07	8.87 ± 0.12	8.75 ± 0.08
9.5	glycine	8.87 ± 0.04	8.83 ± 0.03	8.03 ± 0.04	7.88 ± 0.11
	sarcosine	8.85 ± 0.04	8.75 ± 0.08	8.64 ± 0.10	8.15 ± 0.04
	DMG	8.89 ± 0.05	8.85 ± 0.03	7.44 ± 0.11	7.56 ± 0.12
	betaine	8.79 ± 0.07	8.76 ± 0.11	8.91 ± 0.02	8.80 ± 0.11
	control	8.88 ± 0.03	8.80 ± 0.12	8.95 ± 0.02	8.70 ± 0.02
10.0	glycine	8.34 ± 0.05	6.76 ± 0.09	5.91 ± 0.05	3.58 ± 0.05
	sarcosine	8.52 ± 0.07	7.58 ± 0.05	6.94 ± 0.20	2.46 ± 0.17
	DMG	7.79 ± 0.07	5.34 ± 0.07	2.55 ± 0.16	0.61 ± 1.05
	betaine	8.88 ± 0.07	8.94 ± 0.07	8.91 ± 0.02	8.68 ± 0.03
	control	8.82 ± 0.04	8.98 ± 0.02	8.86 ± 0.08	8.57 ± 0.06

Values represent mean values ± standard deviation of triplicate experiments

Table 6: Summary of *E. coli* culturability at different time points following incubation with saline (control), glycine, sarcosine, DMG and betaine at different pH values



DISCUSSION

An emulsion is described as a heterogeneous liquid system in which one liquid is dispersed in another in the form of droplets (Yokoyama et al., 2001). In order to prepare an emulsion that possesses certain stability, it is necessary to add an emulsifier to the mixture. Surfactants are often used as emulsifiers and / or dispersing agents. It is quite conceivable that amino acids could act as emulsifiers due to their ampholytic nature (Zajic and Panchal, 1976). Of relevance, the pK_a value of the amine group of glycine, sarcosine and DMG presented in table 1, indicates that a larger portion of these molecules will be present in their anionic state with increasing pH, in contrast to betaine.

In apparent contradiction to literature on the emulsifying potential of several betaines, the current results did not confirm this for glycine betaine. The emulsifying potential of DMG has also been described in different contexts (Guan and Tung, 1998; Cools et al., 2010; Kalmar et al., 2010a,b). Kalmar et al. (2010) added DMG to a water-oil mixture and observed a stable emulsion. Our results confirm DMG to be a mild enhancer of emulsification.

Furthermore, when a surfactant accumulates in sufficient quantity it is able to disrupt a lipid bilayer membrane (Xia et al., 2000). Kraft and Moore (2001) state that surfactant molecules adsorbing onto a lipid membrane can induce ion permeability, possibly followed by membrane solubilization. A disrupted ion permeability of the *E. coli* membrane could be responsible for the degeneration of these bacteria upon incubation with glycine, sarcosine and DMG at an alkaline pH, as seen in the current experiments. Some amino acid-based surfactants show antibacterial activity through membrane altering actions (Sánchez et al., 2007). Data on the effect of *N*-methylated derivatives of glycine on bacterial viability are however scarce.

Bacterial viability can be assessed on different levels: bacterial cell growth, structure and metabolism. Growth-based methods, such as the MIC and plate count methods used in our experiments, represent the original concept of bacterial viability solely based on their growth capacity (Sträuber and Müller, 2010). Nevertheless, this approach does not provide detailed information on the effect of glycine, sarcosine, DMG and betaine at the single cell level, nor on the physiological state of the bacteria.

As we aimed to determine such effects of the investigated compounds on the *E. coli* membrane, we used 2-color flow cytometry, based on dual staining with SYTO9 and PI. This allowed to assess membrane integrity as a main and complementary parameter of bacterial viability (Berney et al., 2006 and 2007). The integrity of the cytoplasmic membrane of bacteria is critical in maintaining their viability and metabolic functions, particularly under stress conditions (Mykytczuk et al., 2007).

The observed staining pattern relates to the extent of the membrane damage present. The proportions of intermediate (I) and dead (D) bacterial subpopulations provide an indication of the membrane altering potential of the compounds under the conditions applied. These flow cytometric data clearly show *E. coli* membrane deterioration due to exposure to glycine, sarcosine and DMG, but not betaine.



This effect is both pH- and time-dependent as membrane damage appeared faster at higher pH values, with an onset at pH 9.0. The longer the bacteria were incubated at those conditions, the more severe the induced membrane damage. Although prolonged *in vivo* exposure of *E. coli* to 50 mM of the test compounds at pH 9.0 and higher is unlikely, a clear pH dependent effect of glycine, sarcosine and DMG could be established *in vitro*. Prolonged incubation with saline at the same pH also influenced bacterial viability, this effect was only minor compared to the major influence of glycine, sarcosine and DMG. The decrease in number of culturable bacteria when incubated with glycine, sarcosine or DMG at high alkalinity follows the same trend as the decrease in the percentage of live bacteria shown by flow cytometry. However, in the samples incubated for 20h at pH 10.0 with each of these three test compounds the portion of live bacteria is <1%, while there are still culturable *E. coli* counted after plating. This observation suggests that the subpopulation of intermediate bacteria is heterogeneous consisting of culturable and non-culturable *E. coli*, with varying degrees of membrane damage. The existence of heterogeneity within the intermediate *E. coli* subpopulation was also observed in several other samples exposed to glycine, sarcosine and DMG, and confirms the presence of various viability states within this intermediate population. Another indication for the heterogeneity among the damaged bacteria is the observation of different colony sizes after plating of those samples (data not shown).

Comparing the different test compounds, there are no arguments suggesting that the degree of methylation as such is a key factor in the membrane altering effects. The ability of the compounds to damage the bacterial membrane under alkaline conditions follows the order glycine $\approx$ DMG > sarcosine, with betaine causing no membrane damage under any of the tested conditions. On the contrary, a protective role can even be suggested for betaine.

The flow cytometric data do not concur well with the data gathered on solely emulsifying potential. For DMG the membrane effects could partly be explained by an emulsifying action on the membrane, although the effect of pH on its emulsifying potential was not statistically significant. For glycine and sarcosine no obvious emulsifying potential could be established, while flow cytometry did indicate severe membrane damage due to exposure to these two compounds under alkaline conditions. In contrast, flow cytometric results after 20h of incubation even showed a slightly higher percentage of live bacteria in samples exposed to betaine, compared with the saline treated controls. These findings are in agreement with the lack of emulsifying properties as determined in the current study for betaine. Our CFU counts confirm this positive effect of betaine on *E. coli* survival under prolonged alkaline stress conditions. Although the measured differences are small, they are systematically seen for all pH values tested. The role of betaine as an osmoprotectant for *E. coli* under osmotic stress is well described (Culham et al., 2001; Ly et al., 2004; Tøndervik and Strøm, 2007), but the exact mechanism for its protective role under pH stress is unknown. Nevertheless, there are indications of overlapping mechanisms and roles for osmolytes in osmotic and pH homeostasis (Wang et al., 2007; Kitko et al.,



2010). Betaine is preferably transported into osmotically stressed *E. coli* via the membrane porters ProP and ProU (Csonka, 1989; Peddie et al., 2003). The expression of the ProU gene in *E. coli* encoding for this osmotically inducible transport system is influenced by alkaline changes in cytoplasmic pH (Smirnova and Oktyabrsky, 1995), providing a strong argument for a protective role of betaine under alkaline stress. Interestingly, MacMillan et al. (1999) also describe an inhibitory role for DMG and sarcosine on the uptake of the osmolyte proline by the ProP osmoporter. This finding suggests that both test compounds may also have an effect on *E. coli* membrane pumps that are required to maintain homeostasis under alkaline conditions. Moreover, our results clearly show that alkaline stress alone does not cause *E. coli* membrane damage, or loss of culturability, under the conditions described. It should however be considered that the prolonged incubation of the bacteria in the saline-based test solutions already causes starvation stress. Although our results show no loss of membrane integrity nor culturability due to incubation under these conditions for up to 6h, there is a minor increase of the percentage of intermediates and a decrease in number of culturable bacteria after 20h of incubation in the control samples. Nevertheless even in these 20h incubation samples a clear effect of glycine, sarcosine and DMG could only be seen when incubated at pH 9.0 and higher. This latter observation suggests that starvation stress alone does not make the *E. coli* more susceptible to the effects of the test compounds when incubated for up to 20h. If glycine and its *N*-methylated analogues affect pH homeostasis mechanisms, such as membrane transporters which pump protons inside the bacterial cell to acidify the cytoplasm (Maurer et al., 2005), it can be hypothesized that they will indirectly cause structural damage to these bacteria.

In conclusion, the current results show a decrease in *E. coli* viability due to membrane damage caused by glycine, sarcosine and DMG under alkaline stress conditions, while only DMG could be identified as a weak enhancer of emulsification. In contrast, no emulsifying or membrane altering properties could be established for betaine. Even though the pH-dependent membrane altering effect of glycine, sarcosine and DMG on *E. coli* is clearly demonstrated, the underlying mechanism remains to be elucidated. Questions to be answered are: does the pH-induced altering in ionization state of glycine, sarcosine and DMG molecules enable them to cause direct membrane damage, or does the alkaline pH first effect bacterial homeostasis mechanisms, allowing the latter compounds to affect *E. coli* viability through structural damage, or is it a combination of both effects? This will be the aim of ongoing research to clarify the mechanism of action of glycine and its *N*-methylated analogues.



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Chapter 2

Exposure to the proton scavenger glycine under alkaline conditions induces *Escherichia coli* viability loss

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ABSTRACT

Our previous work described a clear loss of *Escherichia coli* (*E. coli*) membrane integrity after incubation with glycine or its *N*-methylated derivatives *N*-methylglycine (sarcosine) and *N,N*-dimethylglycine (DMG), but not *N,N,N*-trimethylglycine (betaine), under alkaline stress conditions. The current study offers a thorough viability analysis, based on a combination of real-time physiological techniques, of *E. coli* exposed to glycine and its *N*-methylated derivatives at alkaline pH. Flow cytometry was applied to assess various physiological parameters such as membrane permeability, esterase activity, respiratory activity and membrane potential. ATP and inorganic phosphate concentrations were also determined. Membrane damage was confirmed through the measurement of nucleic acid leakage. Results further showed no loss of esterase or respiratory activity, while an instant and significant decrease in the ATP concentration occurred upon exposure to either glycine, sarcosine or DMG, but not betaine. There was a clear membrane hyperpolarization as well as a significant increase in cellular inorganic phosphate concentration. Based on these results, we suggest that the inability to sustain an adequate level of ATP combined with a decrease in membrane functionality leads to the loss of bacterial viability when exposed to the proton scavengers glycine, sarcosine and DMG at alkaline pH.



INTRODUCTION

We recently described a decrease in the viability of stationary phase enterotoxigenic *Escherichia coli* (*E. coli*, *ETEC*) associated with membrane damage and reduced growth capacity caused by glycine and its *N*-methylated derivatives *N*-methylglycine (sarcosine), *N,N*-dimethylglycine (DMG) under alkaline stress conditions (Vanhauteghem et al., 2012). In contrast, the trimethylated analogue of glycine, betaine, did not affect bacterial viability. We now aim to investigate which changes in viability parameters accompany this selective loss of membrane integrity at alkaline pH, providing an indication on the possible underlying mechanism.

Direct membrane interactions of peptides and amino acid-based surfactants causing antibacterial effects are usually related to a net positive charge of these compounds, enhancing their interaction with anionic lipids and other bacterial targets (Jenssen et al., 2006). However, antibacterial effects through membrane altering actions of anionic amino acid-based surfactants have also been described (Sánchez et al., 2006,2007). Besides a direct cytoplasmic membrane effect, it is also possible that the *E. coli* membrane damage occurs secondary to a negative influence on bacterial physiology (Spindler et al., 2011). Indeed, alkaline stress affects bacterial homeostasis mechanisms (Padan et al., 2005), enhancing *E. coli* susceptibility to a disturbance of their functional integrity by glycine, sarcosine and DMG. Fig. 1a represents the physiological conditions (neutral, i.e. no pH stress), where the intracellular pH is kept in a narrow range near pH 7.6 (Booth, 1985; Slonczewski et al., 2009). A shift to an alkaline environment (Fig. 1b) is stressful for bacteria as illustrated by the induction of major stress systems in *E. coli*, such as heat shock responses (Taglicht et al., 1987; Ades et al., 2003), the SOS regulon (Schuldiner et al., 1986), and the Cpx envelope stress mechanisms (DiGiuseppe and Silhavy, 2003). The pH stress also induces several physiological changes (Baatout et al., 2007) and initiates a large number of adaptive strategies. These strategies include: (i) increased metabolic acid production through amino acid deaminases and sugar fermentation; (ii) increased ATP synthase that couples H⁺ entry to ATP generation; (iii) changes in cell surface properties; and (iv) increased expression and activity of monovalent cation/proton antiporters (Padan et al., 2005; Slonczewski et al., 2009). Interference at various levels of the compensation mechanisms may eventually lead to a loss of bacterial viability.

Different complementary approaches can be used to assess bacterial viability/activity (Breeuwer and Abee, 2000; Nebe-von-Caron et al., 2000; Berney et al., 2006; Sträuber and Müller, 2010). In our previous work we established the loss of membrane integrity due to exposure to glycine, sarcosine and DMG under alkaline conditions (Vanhauteghem et al., 2012). These experiments were repeated using log phase *E. coli* and the occurrence of membrane damage was further assessed by measuring the leakage of nucleic (Nobmann et al., 2010). Traditional culture based methods, minimum inhibitory concentrations (MIC) and plate counts, were also performed. The energy status of bacteria is an



important functional indicator of bacterial viability. Bacteria use two forms of metabolic energy electrochemical energy provided by ion gradients and energy-rich phosphate bonds, such as ATP (Breeuwer and Abee, 2000). Measurements of these forms of energy, such as membrane potential and ATP concentration can be used as indicators of cell viability (Sánchez et al., 2010). Next to ATP, the inorganic phosphate (polyP) concentration is also related to the energetic status of bacteria as polyP is considered to be a general source of ATP in several well-known reactions (Brown et al., 2008). Moreover, polyP has also been found to be involved in several bacterial stress responses (Brown et al., 2004 and 2008). Closely connected to the energy metabolism of *E. coli* is its respiratory activity, as the electro-chemical gradient of protons generated by respiration in the process of oxidative phosphorylation is used to synthesize ATP from ADP and inorganic phosphate (Dimroth et al., 2000; Sun et al., 2012). Both the respiratory chain and $F_0 F_1$ -ATP synthase have been reported to regulate the intracellular pH in bacteria (Kinoshita et al., 1984; Kobayashi, 1985). General enzyme activity is another essential factor in maintaining cellular viability. For this purpose, esterases can provide a good indication of the bacterial metabolic activity. They demonstrate the cells' capacity to synthesize these enzymes and maintain them in an active form (Nebe-von-Caron et al., 2000).

The present study performed an in-depth viability analysis based on a combination of real-time physiological techniques which generated novel data allowing the formulation of a clear hypothesis on the effects of glycine, sarcosine and DMG on *E. coli* under alkaline stress conditions, as illustrated in Fig. 1.

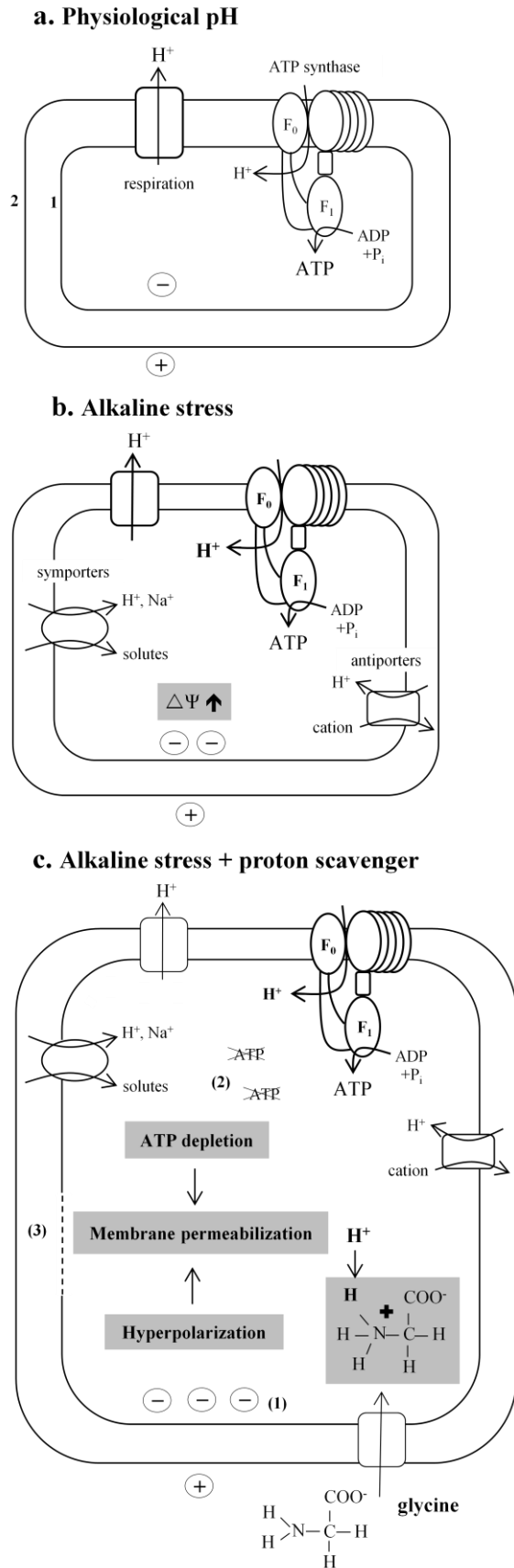


Figure 1:

(a) Maintaining *E. coli* pH homeostasis at physiological pH

During respiration, protons (H^+) are pumped extracellularly, while ATP synthesis via the F_0F_1 -ATP synthase complex moves protons intracellularly. F_0F_1 -ATP synthase converts the free energy of the proton motive force (PMF) into the chemical energy source ATP. Under physiological conditions, the extracellular pH is more acid than the intracellular pH. The cytoplasmic membrane is negatively charged on the inside, and positively charged on the outside. (1) represents the cytoplasmic membrane, (2) represents the outer membrane

(b) Maintaining *E. coli* pH homeostasis under alkaline stress condition

To prevent cytosolic alkalinisation under extracellular alkaline conditions, the cytoplasmic pH is - next to other mechanism - also regulated by the import of protons by the upregulated ATP synthase and by a multitude of cation antiport systems, pumping in protons. The membrane potential ($\Delta\Psi$) is relatively increased (i.e. more negative) to compensate for the inverted proton concentration gradient (ΔpH)

(c) Exposure of alkaline stressed *E. coli* to proton scavenging amines such as glycine (c)

When unprotonated glycine enters the neutral cytosol under extracellular alkaline conditions it becomes protonated. This causes membrane hyperpolarisation (1) by proton consumption and a higher ATP consumption in an effort to sustain pH homeostasis (2). These effects induced by proton scavenging lead to a loss of membrane integrity (3)



MATERIALS AND METHODS

Test Compounds

Glycine ($\geq 99\%$ purity), sarcosine ($\geq 99\%$ purity) and betaine ($\geq 99\%$ purity) were obtained from Sigma Aldrich (St. Louis, MO), DMG ($\geq 97\%$ purity) was obtained from Taminco (Taminco N.V., Ghent, Belgium). All compounds were stored to manufacturers' guidelines until use. Compound characteristics are described by Vanhauteghem et al. (2012).

Bacterial strain, culture and exposure conditions

The haemolytic ETEC strain GIS26, serotype 0149:F4ac, positive for heat labile (LT) and heat stable (STa and STb) enterotoxins (Van den Broeck et al., 1999), was grown overnight at 37°C in brain heart infusion medium (BHI, Oxoid Limited, Hampshire, United Kingdom) to stationary phase. This overnight culture was inoculated 1:100 into fresh BHI broth and grown for 3h to the exponential phase at 37°C. Log phase bacteria were collected by centrifugation (10'000 x g, 2 minutes, room temperature) of 1 ml of the bacterial culture and then resuspended in each of the appropriate test compound solutions in sterile PBS. Initial bacterial concentration was determined by colony plate counts.

A 50 mM solution of each test compound was prepared in sterile PBS. The pH of these solutions was adjusted to pH 9.5 by either HCl or NaOH addition. Log phase *E. coli* were dispersed in 1 ml of each test sample. As a control, pH-adjusted sterile PBS was used. The bacterial suspensions were incubated shaking for 5, 15, 30, 90 and 180 minutes at 37°C. After incubation, analysis of the different viability parameters was performed as described for each parameter below. Proper positive and negative controls were included for each analysis.

Minimum inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) for the test compounds was performed using the broth microdilution assay, using Mueller-Hinton broth at a pH of 6.5, 8.5, 9.0, 9.5 and 10.0. Inoculated microwells free of the test substance, but adjusted to the respective pH values, were included as growth controls, uninoculated microwells were used as sterility controls. Results were recorded after 20h incubation of the microwell plates in an aerobic atmosphere at 35°C ($\pm 2^\circ\text{C}$).

Plate counts

The number CFU per ml was assessed by conventional plate count, which is based on CFU values obtained from a 10-fold serial dilution of each sample plated on Tryptone Soy Agar (Oxoid Limited, Hampshire, United Kingdom) and incubated overnight at 37°C. These plate counts determine the number of culturable bacteria in each sample. All data are the result of triplicate experiments.



Leakage of 260-nm-absorbing material

After incubation samples were centrifuged at 10'000 g for 2 min at 4°C, and 750 µl of the supernatant for each treatment was added to quartz cuvettes and absorbance values at 260nm were recorded using a spectrophotometer (Genesys 10UVn Thermo Electron Company, Cambridge, UK). All experiments were performed in triplicate.

Flow cytometric parameters

All data were obtained using a FACSCanto flow cytometer (Becton, Dickinson and Company, Erembodegem, Belgium). Minimally 10 000 events were recorded and processed using FACS Diva software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). All experiments were performed in triplicate.

Membrane integrity was assessed using the LIVE/DEAD BacLight™ kit (Molecular Probes Eugene, OR, USA) as described by the manufacturer. This bacterial viability kit is widely used in flow cytometry and consists of two nucleic acid stains. Green fluorescent SYTO9 is cell-permeable and can freely enter all *E. coli*, either live or dead. In contrast, red fluorescent propidium iodide (PI) can only enter membrane-compromised cells. Green fluorescence was collected in the FL1 channel (530 ± 15 nm) and red fluorescence in the FL3 channel (>670 nm). All parameters were collected as logarithmic signals. To acquire the correct settings of voltages and compensations different proportions of live and dead bacteria were mixed to obtain cell suspensions containing various ratios. Populations were first single stained (SYTO9 or PI) and subsequently double stained (either SYTO9 and PI mixture). In our set-up, 10 µl of the treated bacterial cell suspension was added to 987 µl of sterile saline. These samples were immediately stained with 3 µl of a mixture of SYTO9 (5 µM final concentration) and PI (30 µM final concentration) and incubated for 15 minutes in the dark at room temperature. Flow cytometric measurements were performed immediately thereafter.

Membrane potential was assessed using the BacLight™ Membrane Potential kit (Molecular Probes Eugene, OR, USA) as described by the manufacturer. The kit contains DiOC₂ which exhibits green fluorescence in all bacterial cells, but the fluorescence shifts toward red emission as the dye molecules self-associate at the higher cytosolic concentrations caused by larger membrane potentials. The red to green fluorescence ratio is used as a size-independent indicator for membrane potential. The proton ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used to provide a depolarized control as it destroys membrane potential by eliminating the proton gradient. In our set-up, 10 µl of the treated bacterial cell suspension was added to 980 µl of sterile PBS. These samples were immediately stained with 10 µl of a DiOC₂ (30 µM final concentration) and incubated for 30 minutes at 37°C in the dark. Flow cytometric measurements were performed immediately thereafter.

Esterase activity was assessed using 5(6)-carboxyfluorescein diacetate (cFDA, Molecular Probes, Eugene, OR, USA). cFDA is an esterified fluorogenic substrate widely used for assessing esterase



activity in bacteria. It is cell permeant and once inside the cell, the non-fluorescent cFDA is enzymatically cleaved via hydrolysis of the diacetate groups by nonspecific esterases into fluorescent carboxyfluorescein (cF), which is accumulated cytosolically (Hoefel et al., 2003). Esterified fluorogenic substrates offer a means of rapid detection of metabolically active bacteria when used in combination with techniques that measure fluorescence at the single cell level, such as flow cytometry. Prior to use, concentrated stock solutions of 21.7 mM cFDA were prepared in DMSO, and further diluted to a final concentration of 10 mM in sterile PBS. Samples of 1 ml of the treated bacterial suspensions were centrifuged ($10'000 \times g$, 2 minutes) and the supernatant was discarded. Cell pellets of *E. coli* were resuspended in 20 μ l of 10 mM cFDA and incubated for 30 min at 37°C in the dark. Following incubation, cells were washed and resuspended in 1 ml sterile PBS and then analyzed by flow cytometry.

Respiratory activity was assessed using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). CTC is a colourless, membrane-permeable compound that produces a red fluorescing precipitate in the cell when it is reduced to its formazan by the electron transport system of bacterial cells (Créach et al., 2003). In our set-up, 20 μ l of the treated bacterial cell suspension was added to 880 μ l of sterile PBS. These samples were immediately stained with 100 μ l of a CTC (5 μ M final concentration) and incubated for 30 minutes at 37°C in the dark. Flow cytometric measurements were performed immediately thereafter.

ATP measurement

For the determination of total ATP, the BacTiter-Glo™ System (Promega, Madison, WI, USA) was used as described by the manufacturer. The BacTiter-Glo™ Buffer was mixed with the lyophilized BacTiter-Glo™ Substrate and equilibrated at room temperature. The mixture was stored for 3h at room temperature to ensure that all ATP was hydrolysed (“burned off”) and the background signal had decreased. A test sample of 100 μ l i.e. both the bacterial population and the incubation medium, was taken from the exposed resuspended bacterial populations and mixed with an equal volume of BacTiter-Glo™ Reagent. Luminescence was measured with a multiplate luminometer (Fluoroscanner Ascent FI, Thermo Labsystems, Helsinki, Finland). A calibration curve with adequate dilutions of pure rATP (Promega, Madison, WI, USA) was measured for each buffer prepared. All data are the result of triplicate experiments.

Inorganic phosphate (polyP) measurement

Intracellular polyP was measured in cell suspension using the DAPI-based fluorescence approach (Aschar-Sobbi et al., 2008). Cells were washed and resuspended in buffer T (100 mM Tris HCl, pH 7.5) and DAPI (4'-6-diamidino-2-phenylindole) (Sigma Aldrich, St. Louis, MO) was added to a final concentration of 10 μ M. After 5 min agitation at 37°C, the DAPI fluorescence spectra (excitation 420



nm with a bandwidth of 8 nm, emission 535 nm with a bandwidth of 25 nm) were recorded using a Perkin Elmer Envision Xcite spectrofluorometer. The fluorescence of the DAPI-polyP complex was used as a measure of intracellular polyP because fluorescence emissions from free DAPI and DAPI-DNA are minimal at this wavelength (Aschar-Sobbi et al., 2008). All data are the result of triplicate experiments.

Statistical analysis

Flow cytometric data of the percentages of live, intermediate and dead bacteria were arcsine-transformed to obtain normal distributions. The CFU counts were logarithmically transformed. ATP, polyP, membrane potential, respiratory activity and leakage at 206 nm data were not transformed. In order to compare the effects of the compounds with the control condition, the Welch' robust variation of ANOVA was used and followed by Dunnett's T3 multiple comparisons. Covariance analysis was performed to compare linear regression slopes of the time course of CFU counts, ATP concentration and flow cytometric based membrane integrity analysis for the different compounds versus control conditions.

RESULTS

MIC determination

Concentrations ranging from 25 mM up to 200 mM of each test compound were investigated for their antibacterial potential against *E. coli* at a pH ranging from 6.5 to 10.0. None of the tested compounds inhibited visible bacterial growth at pH 6.5 to pH 9.0. In contrast, at pH 10.0 all bacterial growth was inhibited due to the highly alkaline pH, as no bacterial growth was observed in the control sample either. At pH 9.5 compound- and concentration-dependent effects occurred. While no inhibition of bacterial growth was yet seen due to exposure to sarcosine, DMG or betaine, glycine inhibited the *E. coli* growth at a concentration of 200 mM. This indicates that the MIC for glycine on the *E. coli* at pH 9.5 lies between 100 and 200 mM, while it exceeds 200 mM for the *N*-methylated analogues tested. The MIC data are presented in Table 1



pH	compound	Concentration (mM)			
		25	50	100	200
6.5	glycine	+	+	+	+
	sarcosine	+	+	+	+
	DMG	+	+	+	+
	betaine	+	+	+	+
	control	+	+	+	+
8.5	glycine	+	+	+	+
	sarcosine	+	+	+	+
	DMG	+	+	+	+
	betaine	+	+	+	+
	control	+	+	+	+
9	glycine	+	+	+	+
	sarcosine	+	+	+	+
	DMG	+	+	+	+
	betaine	+	+	+	+
	control	+	+	+	+
9.5	glycine	+	+	+	-
	sarcosine	+	+	+	+
	DMG	+	+	+	+
	betaine	+	+	+	+
	control	+	+	+	+
10	glycine	-	-	-	-
	sarcosine	-	-	-	-
	DMG	-	-	-	-
	betaine	-	-	-	-
	control	-	-	-	-

Table 1: Overview of the MIC determination data for glycine, sarcosine, DMG, and betaine. The inhibition of visible bacterial growth by concentrations ranging from 25 mM up to 200 mM of each test compound was investigated at a pH ranging from 6.5 to 10.0. The symbol (+) represents visible bacterial growth and (-) represents no visible growth

Plate counts

Complementary to the standard evaluation method of bacterial growth inhibition via the above-described MIC, the bacterial culturability was also assessed through plate counts. Mean values $\pm$ SD of log phase *E. coli* are presented in Fig. 2. Covariance analysis showed a significant difference in linear regression of the control versus glycine and betaine. The slope of the time course of the glycine-exposed sample was significantly more negative than the slope of the control sample ($p = 0.002$). In contrast, the slope of the betaine-exposed sample was more positive compared to the control sample ($p < 0.0005$).

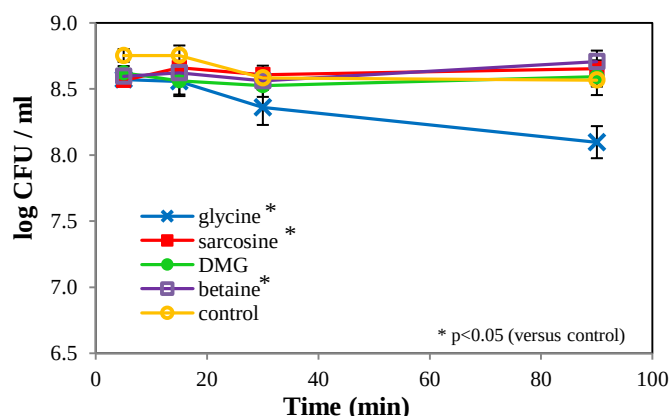


Figure 2: Culturability of *E. coli*. *E. coli* were exposed to glycine, sarcosine, DMG, betaine (50 mM, pH 9.5) and sterile PBS (control, pH 9.5) for up to 90 minutes. The slope of the time course of the glycine-exposed sample is significantly more negative than the slope of the control sample. In contrast, the slope of the betaine exposed sample is more positive compared to the control sample. Data are expressed as means $\pm$ SD of triplicate experiments

Leakage of 260-nm-absorbing material

The presence of nucleic acids and its related compounds, such as pyrimidines and purines, are used as an indicator of cell membrane damage. Absorbance was measured for up to 180 min of *E. coli* exposure to each of the four test compounds (50 mM, pH 9.5), and compared to the control. Mean optical density (OD) values $\pm$ SD are presented in Fig. 3. After 30 min of incubation there was a significant loss of 260-nm-absorbing material upon glycine ($p=0.003$) and DMG ($p=0.006$) incubation. Exposure to sarcosine also led to a significant increased leakage albeit only after 90 min of incubation ($p=0.001$). In contrast, betaine did not show any influence on membrane permeability under these incubation conditions. These results confirm a loss of membrane integrity due to exposure to glycine, and to a lesser and slower extent sarcosine and DMG under alkaline conditions.

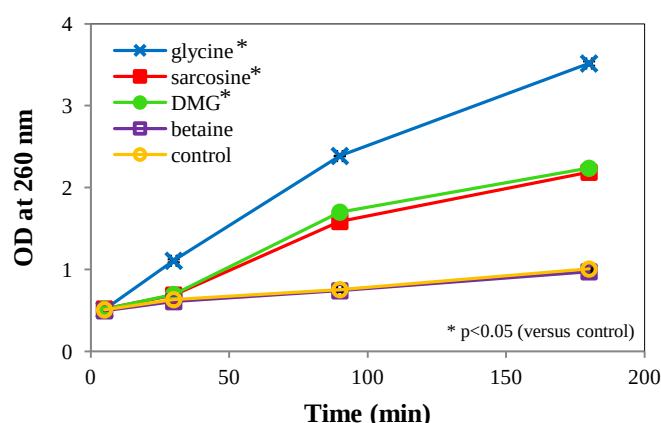


Figure 3: Leakage of 260-nm-absorbing material from *E. coli*. *E. coli* were exposed to glycine, sarcosine, DMG, betaine (50 mM, pH 9.5) and sterile PBS (control, pH 9.5) for up to 180 minutes. After 30 min of incubation there is a significant loss of 260-nm-absorbing material when incubated with glycine ($p=0.003$) and DMG ($p=0.006$). Exposure to sarcosine leads to a significant increased leakage after 90 min of incubation ($p=0.001$). Data are expressed as means $\pm$ SD of triplicate experiments



Flow cytometric assessment of membrane permeability

As previously shown for stationary phase *E. coli* (Vanhautehem et al., 2012), flow cytometric analysis of membrane integrity of log phase *E. coli* with SYTO9/PI dual staining revealed a unique fluorescence pattern, directly related to the degree of membrane damage. Three bacterial subpopulations could thus be identified: membrane-intact live bacteria (A), membrane-damaged “intermediates” (B) and dead bacteria (C). Percentages of these bacterial subpopulations are presented in Fig. 4. Data showed a significant ($p=0.001$) decrease in the mean percentage of live bacteria already after an exposure time of 5 min to glycine (50 mM, pH 9.5) compared to the control. A significant decrease in the percentage of live bacteria after exposure to sarcosine or DMG was observed only after 90 min of incubation ($p= 0.003$ and 0.005 , respectively). Covariance analysis showed a significant difference in linear regression of the control versus glycine, sarcosine and DMG. The slope of the time course of the percentages of membrane-intact live bacteria exposed to the latter 3 compounds was significantly more negative than the slope of the control sample (all $p<0.0005$). Complementary, the slopes of the membrane-damaged intermediate and dead bacteria were significantly more positive compared to the control sample (all $p<0.0005$). Illustrative flow cytometric dot plots of *E. coli* samples incubated with glycine (50 mM, pH 9.5) are presented in Fig. 5.

Overall, these results suggest a compound- and time-dependent onset of membrane damage by glycine versus sarcosine and DMG. In marked contrast, no significant differences in *E. coli* membrane integrity were observed between betaine and the control sample for any of the incubation times measured.

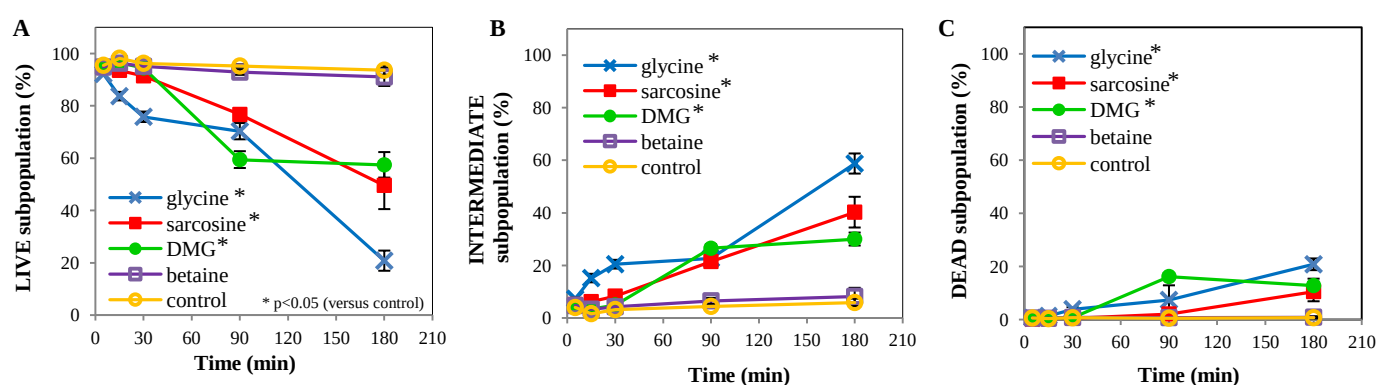


Figure 4: Percentage of membrane-intact live (A), membrane-damaged “intermediates” (B) and irreversibly membrane-damaged dead (C) *E. coli* subpopulations. *E. coli* were exposed to glycine, sarcosine, DMG, betaine (50 mM, pH 9.5) and sterile PBS (control, pH 9.5) for up to 180 minutes. Data are expressed as means $\pm$ SD of triplicate experiments

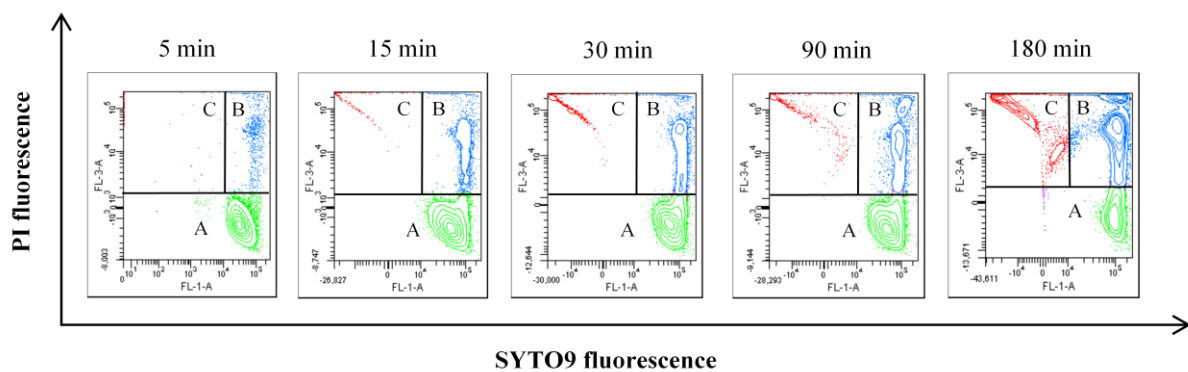


Figure 5: Flow cytometric SYTO9 (FL1)/PI (FL3) dot plots presenting pH-dependent *E. coli* membrane damage. Data obtained for a representative *E. coli* sample incubated with glycine (50 mM, pH 9.5) for up to 3 hours. The A region corresponds to the subpopulation of live cells with an intact plasma membrane, the B region corresponds to the subpopulation of bacteria in an intermediate injured state with different degrees of comprised membranes, the C region corresponds to the subpopulation of dead cells with irreversibly damaged membrane

Flow cytometric assessment of membrane potential

Ratiometric MFI determination of the membrane potential showed a significant hyperpolarization of the *E. coli* membrane after exposure to glycine, sarcosine and DMG (50 mM, pH 9.5) after 90 min of incubation, compared to the control ($p < 0.0005$, $p = 0.002$ and $p < 0.0005$, respectively). In contrast, a time-dependent depolarization of *E. coli* occurred in the betaine-exposed and control samples. Mean ratiometric MFI values $\pm$ SD are presented in Fig. 6.

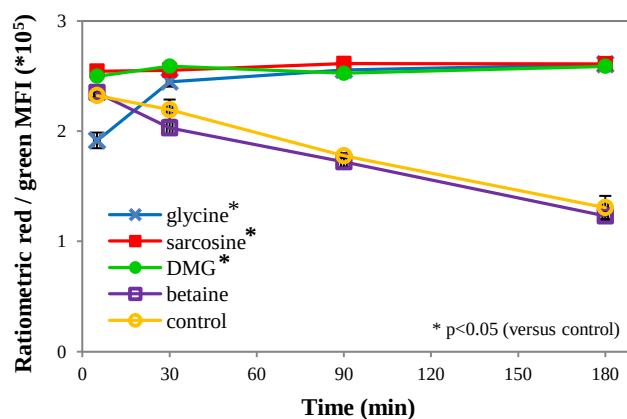


Figure 6: Ratiometric red/green fluorescence presenting *E. coli* membrane potential. Ratiometric membrane potential measurements showed a significant hyperpolarization of the *E. coli* membrane following exposure to glycine, sarcosine and DMG (50 mM, pH 9.5) after 90 min of incubation, compared to the control ($p < 0.0005$, $p = 0.002$ and $p < 0.0005$, respectively). In the betaine-exposed and control samples there is a time-dependent depolarization of *E. coli*. Data are expressed as means $\pm$ SD of triplicate experiments



Flow cytometric measurement of esterase activity

Esterase activity was measured as a marker for the *E. coli* metabolic activity. Fig. 7 shows the representative fluorescence pattern of active and inactive control samples. Inactive cells do not stain (cF⁻) because they either lack enzyme activity and/or cF diffuses freely through the membrane. Metabolically active cells are green fluorescent (cF⁺). Compared to the active control, *E. coli* exposed to only pH 9.5 (control) or to each of the test compounds (50 mM, pH 9.5) showed no decrease in the percentage of metabolically active bacteria for up to 30 min. Overall, these results do not allow to demonstrate a metabolic impairment at the level of enzymatic activity.

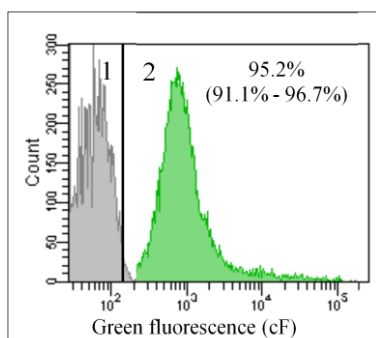


Figure 7: Overlay diagram of the flow cytometric histograms representing metabolically active and inactive *E. coli* subpopulations. Inactive (cF⁻) (1) and active (cF⁺) (2) *E. coli* population are determined by cFDA staining. Inactive cells do not stain because they either lack the enzyme activity to metabolize cFDA to cF and/or cF diffuses freely through the membrane, while metabolically active cells are green fluorescent (cF⁺). Both populations are separated by the black line. The overall mean of triplicates and range are provided

Flow cytometric assessment of respiratory activity

Dehydrogenase activity was measured as a marker for the *E. coli* respiratory activity. Fig. 8A shows the representative fluorescence pattern of active and inactive control samples. Inactive cells (a) do not stain because they lack respiratory dehydrogenase activity. Actively respiring cells (b) become red fluorescent. Fig. 8B presents the MFI of the bacterial populations after exposure to the compounds. Compared to the active control, *E. coli* exposed to glycine and sarcosine (50 mM, pH 9.5) showed a significant decrease in MFI already at 15 min of incubation ($p=0.017$ and $p=0.008$, respectively). This indicates that there is a significant but transient decrease in respiratory activity of the *E. coli* after 15 min of exposure to glycine and sarcosine at pH 9.5. No significant differences were observed between DMG, betaine and the control sample for any of the incubation times measured.

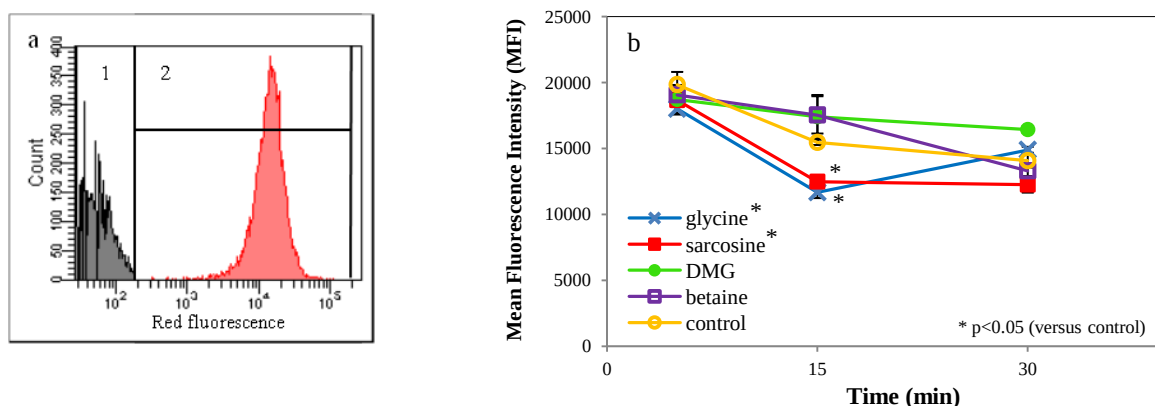


Figure 8:

(a) Overlay diagram of the flow cytometric histograms representing respiratory active and inactive *E. coli* subpopulations. Inactive (1) and active (2) *E. coli* subpopulation are determined by CTC staining. Inactive cells do not stain because they lack respiratory dehydrogenase activity. Actively respiring cells are red fluorescent. Both populations are separated by the black line.

(b) Mean fluorescence intensity (MFI) presenting *E. coli* respiratory activity. Compared to the active control, *E. coli* exposed to glycine and sarcosine (50 mM, pH 9.5) showed a significant decrease in MFI after 15 min of incubation ($p=0.017$ and $p=0.008$, respectively). Data are expressed as means $\pm$ SD of triplicate experiments

ATP Measurement

Intracellular ATP levels were examined for up to 30 min of *E. coli* exposure to each of the four test compounds (50 mM, pH 9.5), and compared to the control. Mean values $\pm$ SD are presented in are presented in Fig. 9.

Cellular ATP depletion started immediately during the incubation with either glycine, sarcosine or DMG. Already after 5 min of incubation a significant (all $p<0.0005$) loss of ATP was observed in these samples, compared to the control. A time-dependent decrease in the ATP concentration occurred also in the control and betaine samples. However, after 30 min of incubation, the ATP level of the bacterial populations exposed to betaine remained significantly ($p=0.001$) higher than the ATP level of the control samples.

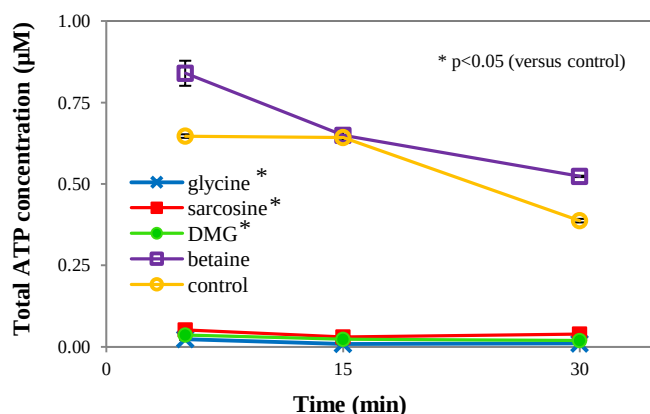


Figure 9: Total ATP concentration of *E. coli*. *E. coli* were exposed to glycine, sarcosine, DMG, betaine (50 mM, pH 9.5) and sterile PBS (control, pH 9.5) for up to 30 min. Already after 5 min of incubation, a significant ($p < 0.0005$) loss of ATP occurs in the bacterial populations exposed to glycine, sarcosine and DMG, compared to the control and betaine sample. Data are expressed as means $\pm$ SD of triplicate experiments

Inorganic phosphate (polyP) measurement

The fluorescence (in arbitrary units) of the DAPI-polyP complex is used as a measure of intracellular polyP. Inorganic phosphate levels were measured for up to 90 min of *E. coli* exposure to each of the four test compounds (50 mM, pH 9.5), and compared to the control. Mean values $\pm$ SD are presented in Fig. 10. After 30 min of incubation there was a significant increase in DAPI-polyP fluorescence when incubated with glycine ($p = 0.009$) compared to the control. Exposure to sarcosine and DMG also led to a significant increase in fluorescence albeit only after 90 min of incubation ($p = 0.03$ and $p = 0.007$, respectively), and not to a level as high as seen after exposure to glycine. In contrast, betaine did not show any influence on polyP concentration under these alkaline incubation conditions.

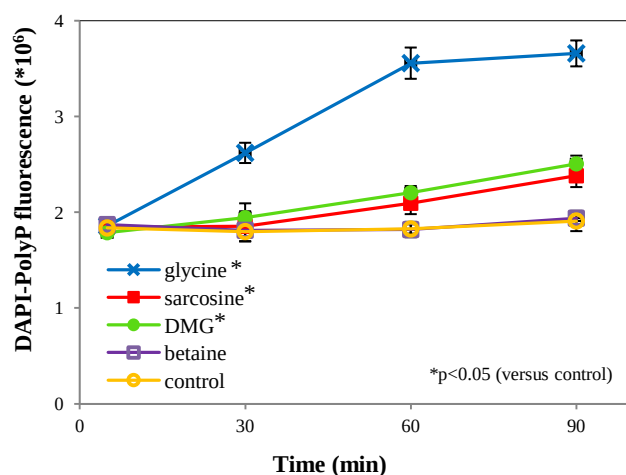


Figure 10: DAPI-polyP fluorescence presenting polyP concentration of *E. coli*.

Inorganic phosphate levels were measured for up to 90 min of *E. coli* exposure to glycine, sarcosine, DMG or betaine (50 mM, pH 9.5), and compared to the control. After 30 min of incubation there is a significant increase in DAPI-polyP fluorescence when incubated with glycine ($p = 0.009$), compared to the control sample. Exposure to sarcosine and DMG leads to a significant increase of fluorescence after 90 min of incubation ($p = 0.03$ and $p = 0.007$, respectively), but not to a level as high as seen after glycine exposure. Betaine exposure had no significant influence compared to the control. Data are expressed as means $\pm$ SD of triplicate experiments



DISCUSSION

Bacterial viability can be assessed at different levels: growth capacity, structural integrity and physiological integrity. Culture-based methods, such as the MIC and plate count methods represent the original concept of bacterial viability, solely based on their growth capacity (Sträuber and Müller, 2010). Our plate count results show a significant linear regression of the CFU after exposure to glycine, sarcosine and DMG (but not betaine), compared to the control (PBS, pH 9.5). This observation, together with the results obtained in previous work (Vanhauteghem et al., 2012), indicates that a prolonged exposure to these former compounds induces a progressive loss of *E. coli* growth capacity under alkaline conditions. Our MIC data only confirmed a growth inhibiting effect for glycine at pH 9.5. However, this latter approach does not provide detailed information on the effects of the compounds at the single cell level, nor on the structural and physiological state of the bacteria.

Essential in bacterial structural integrity is an intact cytoplasmic membrane. We previously described a membrane-damaging effect of glycine, sarcosine and DMG (but not betaine) on stationary phase *E. coli* under alkaline conditions (Vanhauteghem et al., 2012). To ensure an optimal evaluation of the physiological viability parameters described in the present study, we needed to investigate all potential effects on log phase *E. coli*, rather than stationary phase *E. coli*. Our results confirm the loss of membrane integrity, through a nucleic acid flow cytometric staining protocol, and measuring leakage of 260-nm-absorbing material. Both datasets show a significant loss of membrane integrity after 30 min when exposed to glycine, and after 90 min when exposed to sarcosine and DMG. These bacterial populations are still able to grow, as demonstrated by the culture-dependent data. We therefore presume that the initial membrane damage is still reversible, but will progress to irreversible membrane integrity loss upon prolonged exposure (Fig. 1c). This leads to a certain heterogeneity in the bacterial population and a final loss of culturability as demonstrated in our previous work (Vanhauteghem et al., 2012). To provide a detailed assessment of bacterial viability and identify any underlying effects of the compounds, a combination of several viability parameters was further examined.

In metabolically active bacteria with intact cytoplasmic membranes, there is typically a difference of electrical potential across the membrane, with a relatively negative intracellular environment compared to the extracellular environment (Breeuwer and Abee, 2004). Changes in membrane potential are considered an early indicator of injury in bacteria (Hammes et al., 2011). Although a depolarization effect was expected, hyperpolarization has also been reported as an effect associated with bacterial viability loss (Yount et al., 2009; Sánchez et al., 2010; Spindler et al., 2011). Recent studies on this phenomenon suggest that hyperpolarization can occur due to an increase in pH or due to increased movement of ions (Bot and Prodan, 2010). More specifically K^+ diffuses outside the cell through K^+ -channels affecting cellular homeostasis (Bot and Prodan, 2010). Spindler et al. (2011) state



that a non-lethal destabilization of the cytoplasmic membrane can disrupt normal electron flow resulting in at least a transient hyperpolarization of the cytoplasmic membrane. Hyperpolarization is also defined as a higher negative charge at the intracellular side of the cytoplasmic membrane (Castañeda-García et al., 2011), as shown in Fig. 1b. When we consider our compounds in this context, it should be noted that the amine group of glycine, sarcosine and DMG is unprotonated at highly alkaline pH (extracellular) and protonated at a pH near neutrality (intracellular), while the carboxylate group remains negatively charged within this pH range (Fig. 1c) (Vanhauteghem et al., 2012). Thus, once intracellular, the amine group becomes protonated at the near neutral cytoplasmic pH. This causes an alkalization of the cytoplasm and a more negative charge by proton consumption. Of relevance, hyperpolarization can directly comprise membrane integrity (Foster, 2004). Hyperpolarization has also been associated with the formation of superoxide radicals (Spindler et al., 2011), which are implicated in bacterial killing (Kohanski et al., 2007).

The *E. coli* metabolic activity was further evaluated by two complementary parameters: the esterase activity and the total ATP concentration of the bacterial population. Bacterial physiology can only be maintained if the cell is metabolically active and enzymatic reactions, such as those catalyzed by esterases, reflect this activity (Vives-Rego et al., 2000). No loss of bacterial esterase activity occurred either after exposure to each of the compounds or to alkaline stress alone (control, PBS pH 9.5). Therefore, under these conditions *E. coli* could be considered metabolically viable. However, esterase activity was measured for only up to 30 min of incubation, while most bacterial esterases remain stable for a longer period of time, even at high pH values (Gupta et al., 2004). Moreover, it should be emphasized that while enzyme synthesis requires energy, the esterase enzyme-substrate reaction does not, and this enzymatic process can be considered energy-independent (Baatout et al., 2005). True assessment of the energetic state of bacteria is possible by the determination of their ATP level. ATP is the universal energy currency of living cells and as such drives numerous energy-consuming reactions e.g. biosyntheses, mechanical motility, transport through membranes and regulatory networks (von Ballmoos et al., 2009). Indeed, ATP is the most important indicator of the metabolic state and health of cells, including bacteria (Aoki et al., 2009, von Ballmoos et al., 2009). The ATP concentration of the bacterial population decreased significantly and very rapidly after exposure to glycine, sarcosine and DMG (but not betaine) at pH 9.5, compared to the alkaline stress control (Fig. 1c). This instant depletion cannot be attributed to an extracellular leakage of ATP, as our data present both the intracellular and extracellular ATP concentration.

Two pathways exist in *E. coli* for ATP synthesis: glycolysis and oxidative phosphorylation. The F_0F_1 -ATP synthase complex catalyzes the synthesis of ATP from ADP and inorganic phosphate using the electro-chemical gradient of protons generated by respiration during oxidative phosphorylation (Fig. 1a) (Sun et al., 2012). In addition to ATP synthesis, the respiratory chain has been reported to regulate the cytoplasmic pH in *E. coli* (Kinoshita et al., 1984). Krullwich et al. (2011) state that when bacterial



cells are exposed to conditions which require proton influx into the cytoplasm, such as alkaline stress, the expression of the proton pumping $F_0 F_1$ -ATP synthase is elevated in *E. coli*, along with a repression of proton extruding respiratory chain complexes. In line with the latter, our results show a minor, but significant decrease in respiratory activity when the bacteria are exposed to glycine and sarcosine for 15 min. However, this appears to be a transient effect, as respiratory function is already regained at 30 min of incubation.

The $F_0 F_1$ -ATP synthase complex is located in the bacterial cytoplasmic membrane and converts the free energy of the proton motive force (PMF) into the universal chemical energy source ATP (Capaldi et al., 2000). Under physiological conditions (i.e. in the absence of alkaline pH stress), protons move from extracellular to the intracellular, allowing ATP generation (Fig. 1a). In *E. coli*, the PMF driving this synthesis consists of both a transmembrane proton concentration gradient (ΔpH) and an electrical membrane potential ($\Delta \Psi$) component (Kaim and Dimroth, 1998). Both ΔpH and $\Delta \Psi$ are influenced by the extracellular pH (Dimroth et al., 2000), while the $\Delta \Psi$ is additionally modified by several ion membrane transporters (Fig. 1a) (Taglicht et al., 1993). As stated above, the extracellular environment is relatively more acid than the intracellular environment at physiological pH. Under alkaline stress conditions, the situation is reversed which severely influences the PMF (Maurer et al., 2005). The $\Delta \Psi$ increases to compensate for this inverted ΔpH (Fig 1b). Intracellular pH homeostasis in an alkaline environment places a high energy demand on the cell. While numerous responses to pH stress are described, the mechanism by which *E. coli* maintains its cytoplasmic pH near neutrality is very complex and remains only partially understood (Maurer et al., 2005; Krulwich et al., 2011). It is however generally accepted that sustaining this pH homeostasis presents bacteria with a severe bioenergetic challenge. Besides a major influence on bacterial homeostasis, the alkaline conditions also have an important influence on the ionization state of glycine, sarcosine and DMG, but not on that of betaine. The ionization state of the trimethylated analogue betaine remains unaffected. In contrast, the amine group of glycine, sarcosine and DMG is protonated at the near neutral cytoplasmic pH, causing an alkalinization of the cytoplasm by proton consumption (Fig. 1c). A relevant illustration of this proton scavenging mechanism is provided by Yohannes et al. (2005), who report that alkaline pH plays a critical role in polyamine stress. These authors state that the accumulation of polyamines is favored when the cytoplasmic pH is lower than the external pH. Under such conditions, the uncharged base entering the cell is protonated in the cytoplasm and its consumption of protons can impair the ongoing pH homeostasis process (Slonczewski et al., 2009). This implies that glycine, sarcosine and DMG, but not betaine, could also have the potential to amplify pH stress by scavenging protons within the cytoplasm, thus requiring the cell to spend more ATP to support pH homeostasis (Fig. 1c). This increased metabolic energy requirement could explain the very rapid ATP depletion observed in *E. coli* at alkaline pH after incubation with the presumed proton scavengers glycine, sarcosine and DMG in our *in vitro* model. A depletion of the ATP pool has many detrimental consequences, as energy is



required for numerous cellular reactions (Baatout et al., 2005) and can lead to significant membrane damage (Sánchez et al., 2010).

Additionally, our results also show an increase in the inorganic polyphosphate pool (polyP) when the bacteria are exposed to glycine, sarcosine and DMG under alkaline conditions. PolyP can be used in response to a wide variety of metabolic needs and plays central roles in many general physiological processes and even as a buffer against alkaline conditions (Zakharian et al., 2009). It is also involved in the response to several different stress situations and is involved in the SOS response, as well as in the stringent response and in RpoS activation (Brown et al., 2004 and 2008). Although the importance of polyP has been reported for various bacterial species, the precise molecular mechanism by which it enacts specific functions, as well as the primary and secondary effects of polyP accumulation, are still not fully understood in even the best characterized bacterial species (Grillo-Puertas et al., 2012).

In conclusion, we state that exposure to glycine, sarcosine and DMG rapidly induces a marked decrease in *E. coli* viability under alkaline stress conditions. As to the possible mechanism behind this striking viability loss, several assumptions can be made, which all lead to a final loss of membrane integrity. Our data have shown that *E. coli* ATP depletion and membrane hyperpolarization are the major processes preceding severe membrane damage. We provide strong indications that these can be linked to the intracellular proton scavenging effects of glycine, sarcosine and DMG under alkaline conditions.

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Chapter 3

Alkanolamines induce viability loss in *Escherichia coli* and *Pseudomonas oleovorans* subsp. *lubrificantis* at alkaline pH

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ABSTRACT

Our previous study on glycine and its *N*-methylated derivatives generated the hypothesis that these intracellular proton scavengers cause bacterial viability loss in *Escherichia coli* at alkaline pH. The current study aimed to confirm this concept by evaluating the antibacterial potential of monoethanolamine and its *N*-methylated derivatives *N*-monomethylethanolamine (MEA), *N,N*-dimethylethanolamine (DMEA) and *N,N,N*-trimethylethanolamine (choline) under alkaline pH conditions. This parallel series also consists of 3 compounds with an amine group capable of accepting a proton (MEA, MMEA and DMEA) and of 1 compound not able to bind an extra proton (choline). MEA, MMEA and DMEA are described as corrosion inhibitors, with a potential biocidal effect and are used in the metalworking industry. The formulation of metalworking fluids (MWF) makes them highly prone to microbial contamination. Both *E. coli* and *Pseudomonas oleovorans* subsp. *lubricantis* used in this study are important contaminants of MWF. Membrane integrity was evaluated through flow cytometry and measurement of nucleic acid leakage. Culturability was assessed by plate counts and MIC determination. The total ATP concentration of the bacterial populations was also measured. These data confirm our working hypothesis on the antimicrobial effect of certain amines at alkaline pH. Additionally, this study provides novel evidence on both the efficacy and probable working mechanism of alkanolamines as antimicrobial compounds in MWF. The *E. coli* populations showed an immediate ATP depletion, followed by membrane permeabilization and loss of culturability when exposed to MEA, MMEA and DMEA under alkaline conditions. Choline had no effect on ATP concentration and culturability. Results on *P. oleovorans* also showed a clear antibacterial effect, but now without a concomitant significant decrease in ATP concentration. This latter implies that either *P. oleovorans* is more resilient to perturbations of its cellular ATP metabolism than *E. coli*, and/or that the underlying mechanism of action does differ from the one postulate for *E. coli*.



INTRODUCTION

The metalworking industry utilizes recirculating MWF to cool, remove metal fines, lubricate and prevent corrosion during metal grinding and cutting procedures (Liu et al., 2010). Metalworking fluids are complex mixtures of oils, biocides, dissolved metals, antifoaming agents and many other organic and inorganic components (Sandin et al., 1991; Perkins and Angenent, 2010). They are formulated to improve longevity of equipment, but their formulation also makes them highly prone to physical, chemical and microbial contamination (Laitinen et al., 1999; Saha et al., 2012). Uncontrolled microbial growth can significantly impact both the performance of these MWF and the health of the machine operators (Wallace et al., 2002; Perkins and Angenent, 2010; Selvaraju et al., 2011). Microbial degradation of MWF can result in corrosion of machines, tools and work pieces, in loss of lubricity and fluid instability, in decrease of the fluid pH (organic acid production), in slime formation (so-called biofilms), which can plug filters, causes unacceptable odors and carries important health risks for operators (Rossmore and Rossmore, 1991; Bakalova et al., 2007). On the other hand, the MWF deterioration capacity of microorganisms may also provide an important solution towards a safe and economical disposal of operationally exhausted MWF (van der Gast et al., 2001, 2002; van der Gast and Thompson, 2004).

Microbial contamination of MWF is inherently related to their composition. Foxall-VanAken et al. (1986) already stated that 3 important classes of compounds are usually present in MWF at a sufficient concentration to support microbial growth: oil, petroleum sulphonates and fatty acids. Next to these key carbon sources, breakdown products of other microorganisms may also serve as nutrients (Laitinen et al., 1999). However, the highly selective nature of the substrate limits the microbial diversity in MWF (Mattsby-Baltzer et al., 1989; van der Gast et al., 2001, 2003). It is the general chemistry of a MWF, rather than any particular component hereof, which selects for the microbial community composition (van der Gast et al., 2003). Many studies identify pseudomonads as the predominant species (Sondossi et al., 1984; Mattsby-Baltzer et al., 1989; van der Gast et al., 2001; Gilbert et al., 2010; Perkins and Angenent, 2010; Saha and Donofrio, 2012). A variety of other bacterial classes has also been described as being abundant in MWF (Laitinen et al., 1999; Bakalova et al., 2007; Gilbert et al., 2010; Murat et al., 2011; Saha and Donofrio, 2012).

The use of biocides is the most common strategy for controlling microbial growth in MWF, with formaldehyde condensates being the most popular chemical agent (Rossmore, 1995; Wallace et al., 2002). Nevertheless, the use (of combinations) of biocides is subjected to severe regulations and limitations. Consequently, there is an ongoing search for novel MWF biocides providing superior alternatives. For this purpose it is important to evaluate each candidate biocide against appropriate problem strains, both in controlled and MWF matrix conditions (Selvaraju et al., 2005). Indeed, as an



initial screening, the individual potential of candidate compounds should be verified in a controlled environment. The selection of MWF representative bacterial species is also of great relevance.

Alkanolamines are used in MWF as corrosion inhibitors, but they have also been previously described for their potential as biocide in these MWF (Bennett et al., 1979; Bakalova et al., 2008). More than 2 decades ago, Sandin et al. (1990, 1991 and 1992) already showed that alkanolamines have an antibacterial effect, which is greatly enhanced at alkaline pH. In this study we evaluated the effect of a structurally related series of alkanolamines on *Pseudomonas oleovorans* subsp. *lubricantis*, a novel species described by Saha et al. (2010) and previously referred to as *P. oleovorans* or *P. pseudoalcaligenes*. We systematically compared this to the effects on *E. coli*, as we previously described an antibacterial effect for a related series of glycine and its *N*-methylated amine derivatives on this pathogen under alkaline stress conditions (Vanhautehem et al., 2013). Of relevance, *E. coli* species are also described as possible microbial contaminants of MWF, often introduced by machine operators and/or by environmental contamination (Gilbert et al., 2010; Lucchesi et al., 2012; Saha and Donofrio, 2012).

In the current study, we now aim to validate our previous hypothesis by testing the antibacterial effect of MEA and its mono- and di-*N*-methylated derivatives MMEA and DMEA. To further confirm the importance of the capacity for intracellular proton scavenging which underlies this hypothetical antibacterial mechanism. We also included choline, being the trimethylated derivative of MEA and the homologue of betaine in our previously tested glycine series. The pK_a values of all 4 test compounds are presented in Table 1. Choline cannot bind an extra proton on its amine group and we therefore hypothesize here that it should not exhibit antibacterial properties under alkaline stress conditions.



	structure	pK _a
MEA		9.50
MMEA		9.95
DMEA		9.23
choline		13.90

MEA = ethanolamine; MMEA = methylethanolamine;
DMEA = dimethylethanolamine

Table 1: Chemical structures and pK_a values of monoethanolamine and its mono-, di- and tri-*N*-methylated analogues

MATERIALS AND METHODS

Test Compounds

Monoethanolamine (MEA) was obtained from Sigma Aldrich (St. Louis, MO), methylmonoethanolamine (MMEA), dimethylethanolamine (DMEA) and choline were obtained from Taminco (Taminco B.V.B.A., Ghent, Belgium). All compounds were stored to manufacturers' guidelines until use.

Bacterial strain, culture and exposure conditions

The haemolytic ETEC strain GIS26, serotype 0149:F4ac, positive for heat labile (LT) and heat stable (STa and STb) enterotoxins (Van den Broeck et al., 1999), was grown overnight at 37°C in brain heart infusion medium (BHI, Oxoid Limited, Hampshire, United Kingdom) to stationary phase. This overnight culture was inoculated 1:100 into fresh BHI broth and grown for 3h to the exponential phase at 37°C. Log phase bacteria were collected by centrifugation (10'000 x g, 2 minutes, room temperature) of 1 ml of the bacterial culture and then resuspended in each of the appropriate test compound solutions in sterile PBS. Initial bacterial concentration was determined by colony plate counts.



Pseudomonas oleovorans subsp. *lubricantis* (ATCC BAA-1494) was obtained from the DSMZ culture collection (Germany). It was grown for 24h at 37°C in Müller-Hinton broth (MH, Oxoid Limited, Hampshire, United Kingdom). Log phase bacteria were collected by centrifugation (10'000 x g, 2 minutes, room temperature) of 5 ml of the bacterial culture and then resuspended in each of the appropriate test compound solutions in sterile PBS. Initial bacterial concentration was determined by colony plate counts.

A 50 mM solution of each test compound was prepared in sterile PBS. The pH of these solutions was adjusted to pH 9.5 by either HCl or NaOH addition. Bacteria were dispersed in 1 ml of each test sample. As a control, pH-adjusted sterile PBS was used. The bacterial suspensions were incubated shaking at 37°C. After incubation, analysis of the different viability parameters was performed as described for each parameter below. Proper positive and negative controls were included for each analysis.

Minimum inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) for the test compounds was performed using the broth microdilution assay, using Müller-Hinton broth at a pH of 6.5, 8.5, 9.0, 9.5 and 10.0. Inoculated microwells free of the test substance, but adjusted to the respective pH values, were included as growth controls, uninoculated microwells were used as sterility controls. Results were recorded after 20h incubation of the microwell plates in an aerobic atmosphere at 35°C (+/-2°C).

Plate counts

Log phase *E. coli* were incubated with the test compound solutions for up to 180 min, while stationary phase *E. coli* and log phase *P. oleovorans* were exposed for up to 300 min. The number CFU per ml was assessed by conventional plate count, which is based on CFU values obtained from a 10-fold serial dilution of each sample plated on Tryptone Soy Agar (Oxoid Limited, Hampshire, United Kingdom) and incubated overnight at 37°C. These plate counts determine the number of culturable bacteria in each sample. All data are the result of triplicate experiments.

Assessment of membrane permeability

Log phase *E. coli* were incubated with the test compound solutions for up to 180 min, while stationary phase *E. coli* and log phase *P. oleovorans* were exposed for up to 300 min.

Leakage of 260-nm-absorbing material

After incubation samples were centrifuged at 10'000 g for 2 min at 4°C, and 750 µl of the supernatant for each treatment was added to quartz cuvettes and absorbance values at 260nm were recorded using



a spectrophotometer (Genesys 10UVn Thermo Electron Company, Cambridge, UK). All experiments were performed in triplicate.

Flow cytometric membrane integrity measurement

All data were obtained using a FACSCanto flow cytometer (Becton, Dickinson and Company, Erembodegem, Belgium). Minimally 10 000 events were recorded and processed using FACS Diva software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). All experiments were performed in triplicate.

Membrane integrity was assessed using the LIVE/DEAD BacLight™ kit (Molecular Probes Eugene, OR, USA) as described by the manufacturer. This bacterial viability kit is widely used in flow cytometry and consists of two nucleic acid stains. Green fluorescent SYTO9 is cell-permeable and can freely enter all *E. coli*, either live or dead. In contrast, red fluorescent propidium iodide (PI) can only enter membrane-compromised cells. Green fluorescence was collected in the FL1 channel (530 ± 15 nm) and red fluorescence in the FL3 channel (>670 nm). All parameters were collected as logarithmic signals. To acquire the correct settings of voltages and compensations different proportions of live and dead bacteria were mixed to obtain cell suspensions containing various ratios. Populations were first single stained (SYTO9 or PI) and subsequently double stained (either SYTO9 and PI mixture). In our set-up, 10 μ l of the treated bacterial cell suspension was added to 987 μ l of sterile saline. These samples were immediately stained with 3 μ l of a mixture of SYTO9 (5 μ M final concentration) and PI (30 μ M final concentration) and incubated for 15 minutes in the dark at room temperature. Flow cytometric measurements were performed immediately thereafter.

ATP measurement

Log phase *E. coli* were incubated with the test compound solutions for up to 60 min, while stationary phase *E. coli* and log phase *P. oleovorans* were exposed for up to 120 min. For the determination of total ATP, the BacTiter-Glo™ System (Promega, Madison, WI, USA) was used as described by the manufacturer. The BacTiter-Glo™ Buffer was mixed with the lyophilized BacTiter-Glo™ Substrate and equilibrated at room temperature. The mixture was stored for 3h at room temperature to ensure that all ATP was hydrolysed (“burned off”) and the background signal had decreased. A test sample of 100 μ l i.e. both the bacterial population and the incubation medium, was taken from the exposed resuspended bacterial populations and mixed with an equal volume of BacTiter-Glo™ Reagent. Luminescence was measured with a multiplate luminometer (Fluoroscan Ascent FI, Thermo Labsystems, Helsinki, Finland). A calibration curve with adequate dilutions of pure rATP (Promega, Madison, WI, USA) was measured for each buffer prepared. All data are the result of triplicate experiments.



Statistical analysis

Flow cytometric data of the percentages of live, intermediate and dead bacteria were arcsine-transformed to obtain normal distributions. The CFU counts were logarithmically transformed. ATP and leakage at 206 nm data were not transformed. In order to compare the effects of the compounds with the control condition, the Welch' robust variation of ANOVA was used and followed by Dunnett's T3 multiple comparisons. Covariance analysis was performed to compare linear regression slopes of the time course for the different compounds versus control conditions.

RESULTS AND DISCUSSION

MIC determination

Concentrations ranging from 25 mM up to 200 mM of each test compound were investigated for their antibacterial potential against *E. coli* and *P. oleovorans* at a pH ranging from 6.5 to 10.0 (Table 2).

For *E. coli* none of the compounds inhibited visible bacterial growth at pH 6.5 and 7.5 for the tested concentrations. In contrast, at pH 10.0 all bacterial growth was inhibited due to the highly alkaline pH, as no bacterial growth was observed in the control sample either. At pH 8.5 to 9.5 compound- and concentration-dependent effects occurred. Results show that the MIC for MEA and MMEA at pH 8.5, 9.0 and 9.5 lie between 100 and 200 mM, between 50 and 100 mM and between 25 and 50 mM, respectively. The MIC for DMEA exceeds 200 mM at pH 8.5 and is situated between 100 and 200 mM, and between 25 and 50 mM at pH 9.0 and 9.5, respectively. Choline surprisingly also inhibited *E. coli* growth, albeit only at a high concentration of 200 mM and only at pH 9.5.

For *P. oleovorans* an antibacterial effect was seen at all pH values tested. In marked contrast to *E. coli*, already at pH 6.5 and 7.5 bacterial growth was decreased, respectively inhibited due to exposure to 200 mM MEA and MMEA. At pH 8.5 the MIC lies between 50 and 100 mM for MEA and MMEA, and between 100 and 200 mM for DMEA. At pH 9.0 and 9.5 the MIC lies between 25 and 50 mM for MEA, and between 50 and 100 mM for MMEA and DMEA. Again in marked contrast to *E. coli*, pH 10.0 did not inhibit bacterial growth in the control sample, indicating that this very high alkaline pH is not inhibitory to *P. oleovorans*. At this latter pH the MIC for MEA, MMEA and DMEA lies between 25 and 50 mM. Choline did not inhibit *P. oleovorans* growth under any of the conditions tested.

Our MIC data show a difference in susceptibility between both Gram negative bacteria *E. coli* and *P. oleovorans*: while *E. coli* incubation at alkaline pH is required for MEA, MMEA and DMEA to have a growth inhibiting effect, for *P. oleovorans* growth is already inhibited at near neutral pH when exposed to the highest concentration (200 mM) of MEA and DMEA. Overall, these MIC data suggest that the *P. oleovorans* better withstands the antibacterial effects of MEA, MMEA and DMEA at alkaline pH than the *E. coli*.



pH	compound	<i>E. coli</i>				<i>P. oleovorans</i>			
		Concentration (mM)				Concentration (mM)			
		25	50	100	200	25	50	100	200
6.5	MEA	+	+	+	+	+	+	+	±
	MMEA	+	+	+	+	+	+	+	±
	DMEA	+	+	+	+	+	+	+	+
	choline	+	+	+	+	+	+	+	+
	control	+	+	+	+	+	+	+	+
7.5	MEA	+	+	+	+	+	+	+	-
	MMEA	+	+	+	+	+	+	+	-
	DMEA	+	+	+	+	+	+	+	+
	choline	+	+	+	+	+	+	+	+
	control	+	+	+	+	+	+	+	+
8.5	MEA	+	+	+	-	+	+	-	-
	MMEA	+	+	+	+	+	+	-	-
	DMEA	+	+	+	+	+	+	+	-
	choline	+	+	+	+	+	+	+	+
	control	+	+	+	+	+	+	+	+
9.0	MEA	+	+	-	-	+	-	-	-
	MMEA	+	+	-	-	+	+	-	-
	DMEA	+	+	+	-	+	+	-	-
	choline	+	+	+	+	+	+	+	+
	control	+	+	+	+	+	+	+	+
9.5	MEA	+	-	-	-	+	±	-	-
	MMEA	+	-	-	-	+	±	-	-
	DMEA	+	-	-	-	+	±	-	-
	choline	+	+	+	-	+	+	+	+
	control	+	+	+	+	+	+	+	+
10.0	MEA	-	-	-	-	±	-	-	-
	MMEA	-	-	-	-	±	-	-	-
	DMEA	-	-	-	-	+	-	-	-
	choline	-	-	-	-	+	+	+	+
	control	-	-	-	-	+	+	+	+

Table 2: Overview of the MIC data for MEA, MMEA, DMEA and choline compared to the control (phosphate-buffered saline). The inhibition of visible bacterial growth by concentrations ranging from 25 mM up to 200 mM of each test compound was investigated at a pH ranging from 6.5 to 10.0. The symbol (+) represents visible bacterial growth, while the symbol (-) represents no visible growth and (±) represents a less dense bacterial culture, suggesting delayed growth



Plate counts

Complementary to the standard evaluation method of bacterial growth inhibition via the above-described MIC, the bacterial culturability was also assessed through plate counts. Mean values $\pm$ SD of bacterial counts are presented in Fig. 1.

It should be remarked that for *E. coli* there is a significant difference between log and stationary phase bacteria. Log phase *E. coli* exposed to MEA, MMEA and DMEA (50 mM, pH 9.5) already showed a significant decrease in the number of culturable bacteria after 30 min of exposure ($p=0.002$, $p<0.0005$ and $p=0.001$ respectively), while the number of CFU obtained from stationary phase *E. coli* decreased significantly only after 60 min of incubation with DMEA ($p=0.013$) and 180 min with MEA and MMEA ($p=0.012$ and $p=0.009$ respectively). Choline had no effect on the number of culturable *E. coli* in either log or stationary phase.

For *P. oleovorans* only log phase bacterial cultures were tested. Results showed a significant decrease in culturability when exposed to MEA and MMEA (50 mM, pH 9.5) after 60 min of incubation ($p=0.025$ and $p=0.040$ respectively) and instantly (already after 5 min) when exposed to DMEA ($p=0.023$). Unexpectedly, incubation with choline induced a significant increase of the number of viable *P. oleovorans* bacteria after 180 min of incubation ($p=0.013$). Covariance analysis showed a significant difference in linear regression of the control versus all test compounds. The slope of the time course of the MEA-, MMEA- and DMEA-exposed samples was significantly more negative than the slope of the control sample ($p<0.0005$ for all). In contrast, the slope of the choline-exposed sample was more positive compared to the control sample ($p=0.013$).

Corroborating literature, a difference was observed between log phase and stationary phase *E. coli*. Several authors indeed described that log phase *E. coli* are less resistant to stress conditions and lose culturability more quickly than stationary phase cells (Munro et al., 1995; Dantur and Pizarro, 2004). Moreover, bacteria develop several morphological and physiological changes as they progress into stationary phase displaying an increased resistance to stress (Vulic and Kolter, 2002).

Compared to *E. coli* our data furthermore indicate a higher resilience of *P. oleovorans* to the effects of MEA, MMEA and DMEA on culturability. This could be explained by the fact that *Pseudomonas* spp. have much simpler nutrient requirements for their survival and a higher genetic and metabolic adaptability to stress situations, making them very resilient (Arana et al., 2010). For *E. coli* on the other hand, the very poor (with respect to nutrients) phosphate-buffered saline-based incubation conditions, such as used in our experimental set-up, represent a much more hostile environment (Arana et al., 2010).

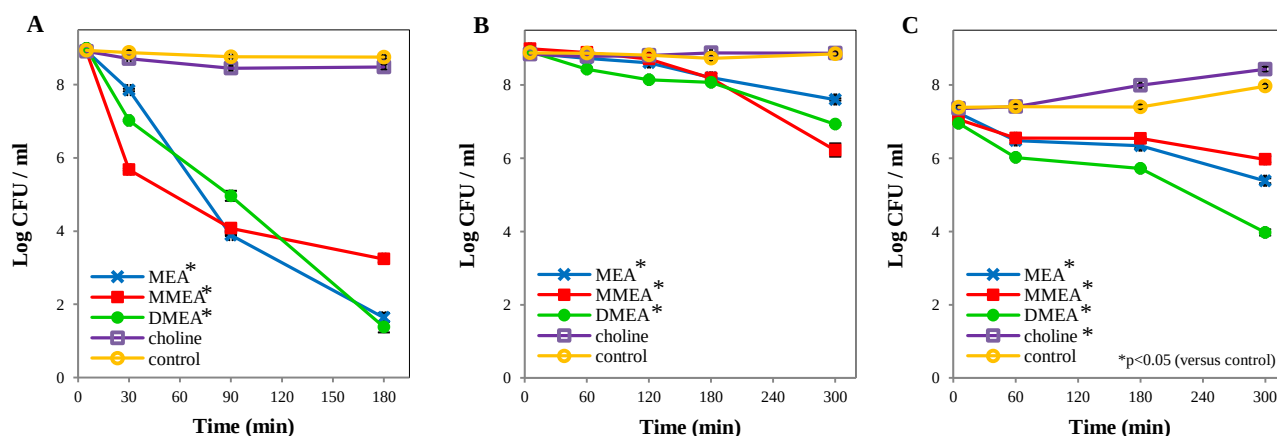


Figure 1: Culturability of log phase *E. coli* (A), stationary phase *E. coli* (B) and log phase *P. oleovorans* (C). Bacterial populations were exposed to MEA, MMEA, DMEA, choline (50 mM, pH 9.5) and sterile PBS (control, pH 9.5). Data are expressed as means $\pm$ SD of triplicate experiments

Assessment of membrane permeability

Leakage of 260-nm-absorbing material

The presence of nucleic acids and their building blocks, such as pyrimidines and purines, are used as an indicator of cell membrane damage. Absorbance was measured at different time points during exposure to each of the 4 test compounds (50 mM, pH 9.5) and compared to the control. Mean optical density (OD) values $\pm$ SD are presented in Fig. 2.

For *E. coli*, both log and stationary phase bacteria were again used. For log phase bacteria a significant increase in absorbance was observed after 30 min of incubation with MEA, MMEA and DMEA ($p < 0.0005$ for all 3 compounds). The leakage of 260-nm-absorbing material further increased with time for these 3 compounds. When log phase *E. coli* were exposed to choline a significant - albeit to a far less extend - increase in leakage was only seen after 90 min of incubation ($p = 0.020$). For stationary phase *E. coli*, a similar pattern was seen: a significant increase in membrane permeability after 60 min of exposure to MEA, MMEA and DMEA ($p < 0.0005$ for all) and after 180 min of exposure to choline ($p = 0.002$).

For *P. oleovorans* again only log phase bacteria were used. After 180 min of incubation there was a significant loss of 260-nm-absorbing material when incubated with MEA, MMEA and DMEA ($p < 0.0005$ for all 3 compounds). However, when compared to *E. coli*, the absolute OD values were much lower. Choline did not show any influence on membrane permeability of *P. oleovorans* under these incubation conditions.



These data confirm the difference seen with plate counts between log and stationary phase *E. coli*. Significant membrane permeabilization occurs much faster in the cultures grown to an exponential state, which could be explained by a difference in stress resistance (Vulic and Kolter, 2002). An alternative explanation might be found in the difference in membrane fluidity of both types of bacterial cultures. *E. coli* can adapt their membrane fatty acid and phospholipid composition under environmental stress situations and exposure to toxic compounds (Pedrotta and Witholt, 1999; Härtig et al., 2005). An increase in the saturation degree of the membrane lipids results in a more rigid, and thus more resistant, cytoplasmic membrane. Härtig et al. (2005) state that stationary phase bacteria have a higher degree of membrane lipid saturation than log phase bacteria, suggesting that these former populations have a more resistant membrane. This could at least partly explain the later onset of membrane permeabilization in stationary compared to log phase *E. coli*.

Finally, these data again indicate a difference between *E. coli* and *P. oleovorans* with respect to the effect of MEA, MMEA, DMEA and even choline, now situated on the level of membrane permeability. More specially, *P. oleovorans* appears to be more resilient to membrane altering effects resulting from the exposure to MEA, MMEA or DMEA. Interestingly, literature does not suggest a relevant difference between the *E. coli* and *P. oleovorans* cytoplasmic membrane.

We previously proposed the membrane damage to be a secondary effect. Less membrane damage could therefore indicate that the primary damage induced in *P. oleovorans* is not as severe as that in *E. coli*, like suggested by Arana et al. (2010).

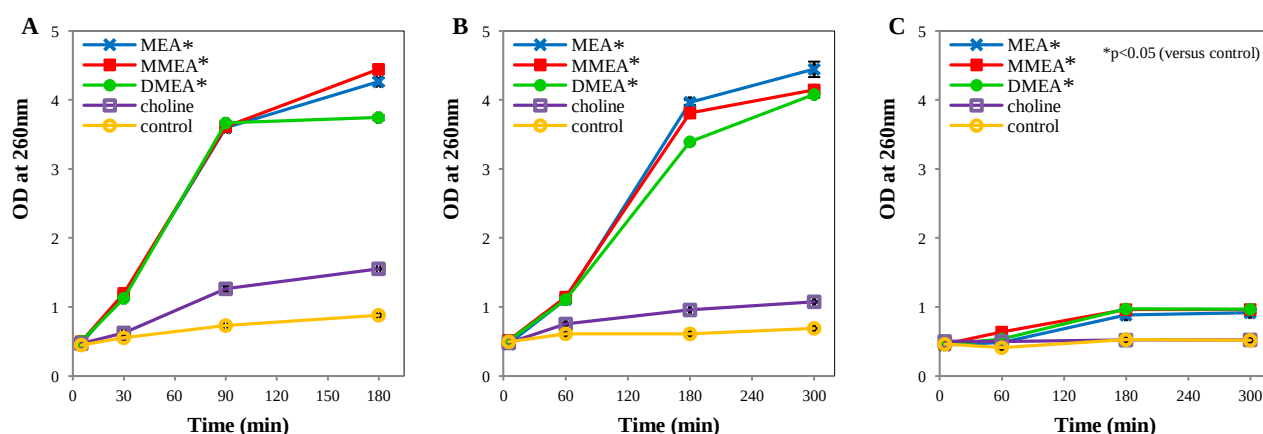


Figure 2: Leakage of 260-nm-absorbing material from log phase *E. coli* (A), stationary phase *E. coli* (B) and log phase *P. oleovorans* (C). Bacterial populations were exposed to MEA, MMEA, DMEA, choline (50 mM, pH 9.5) and sterile PBS (control, pH 9.5). Data are expressed as means $\pm$ SD of triplicate experiments



Flow cytometric assessment of membrane permeability

As previously shown for *E. coli* (Vanhautehem et al., 2012) flow cytometric analysis of membrane integrity with SYTO9/PI dual staining used in the current study revealed a unique fluorescence pattern which is directly related to the degree of membrane damage. Three bacterial subpopulations could thus be identified: membrane-intact live bacteria (A), membrane-damaged “intermediates” (B) and dead bacteria (C). The percentages of these bacterial subpopulations are presented in Fig. 3.

For *E. coli* there was again a significant difference between the effect of the test compounds on stationary and log phase bacteria. When log phase *E. coli* were exposed to MEA, MMEA and DMEA (50 mM, pH 9.5) an instant (already after 5 min) significant decrease in percentage of live bacteria ($p=0.001$, $p<0.0005$ and $p<0.0005$, respectively) was seen. After 180 min of incubation >90% of the bacterial population was even classified as dead. In contrast, incubation with choline only caused a mild ($p=0.036$) increase in the percentage of intermediate bacteria.

When stationary phase *E. coli* were exposed to MEA, MMEA, DMEA similar effects were seen, albeit delayed as they occurred after longer incubation times. For all 3 compounds there was again an instant (already after 5 min) significant decrease in the percentage of live bacteria ($p=0.001$, $p=0.001$ and $p=0.011$, respectively). However, even after 300 min of incubation, the percentage of dead bacteria was not yet as high as seen with log phase *E. coli* after only 180 min of incubation. In marked contrast, choline did not cause a significant increase in membrane permeabilisation as determined by flow cytometry. Overall, these results suggest a compound- and time-dependent onset of membrane damage by exposure of *E. coli* to MEA, MMEA and DMEA, with a higher susceptibility in log phase bacteria. Choline does not have a significant effect, even though leakage of 260-nm-absorbing material was observed.

For *P. oleovorans* again only log phase bacteria were exposed to MEA, MMEA, DMEA and choline (50 mM, pH 9.5). Results showed an instant (already after 5 min) significant decrease in the percentage of live bacteria when exposed to MEA, MMEA and DMEA ($p<0.0005$, $p=0.018$ and $p=0.002$, respectively). However, these membrane-compromised bacteria remained in the intermediate state even after incubation for 300 min. After this prolonged exposure there was no significant increase in the percentage of dead bacteria for any of the test compounds. Moreover, no significant differences in membrane integrity were observed between choline and the control sample after any of the incubation times evaluated. These data confirm our membrane permeability data obtained by the measurement of 260-nm-absorbing material leakage. The decrease in culturable bacteria indicates that the intermediate population is heterogeneous, as some of the intermediates are still culturable, while others are no longer able to grow.

Overall, these results confirm the membrane permeability data at three levels: (i) there is a loss of *E. coli* membrane integrity due to exposure to MEA, MMEA and DMEA at alkaline pH, which is comparable to, but more severe than the effects that were previously seen after incubation with



glycine, sarcosine and DMG under the same conditions (Vanhauteghem et al., 2012, 2013); (ii) log phase *E. coli* are less resistant than stationary phase *E. coli*, this effect is seen in both the glycine series (Chapter 1, i.e. stationary phase *E. coli* and 2, i.e. log phase *E. coli*), and the current data with the monoethanolamine series of compounds; (iii) *P. oleovorans* appears to be more resilient than *E. coli* to membrane altering effects resulting from the exposure to MEA, MMEA or DMEA.

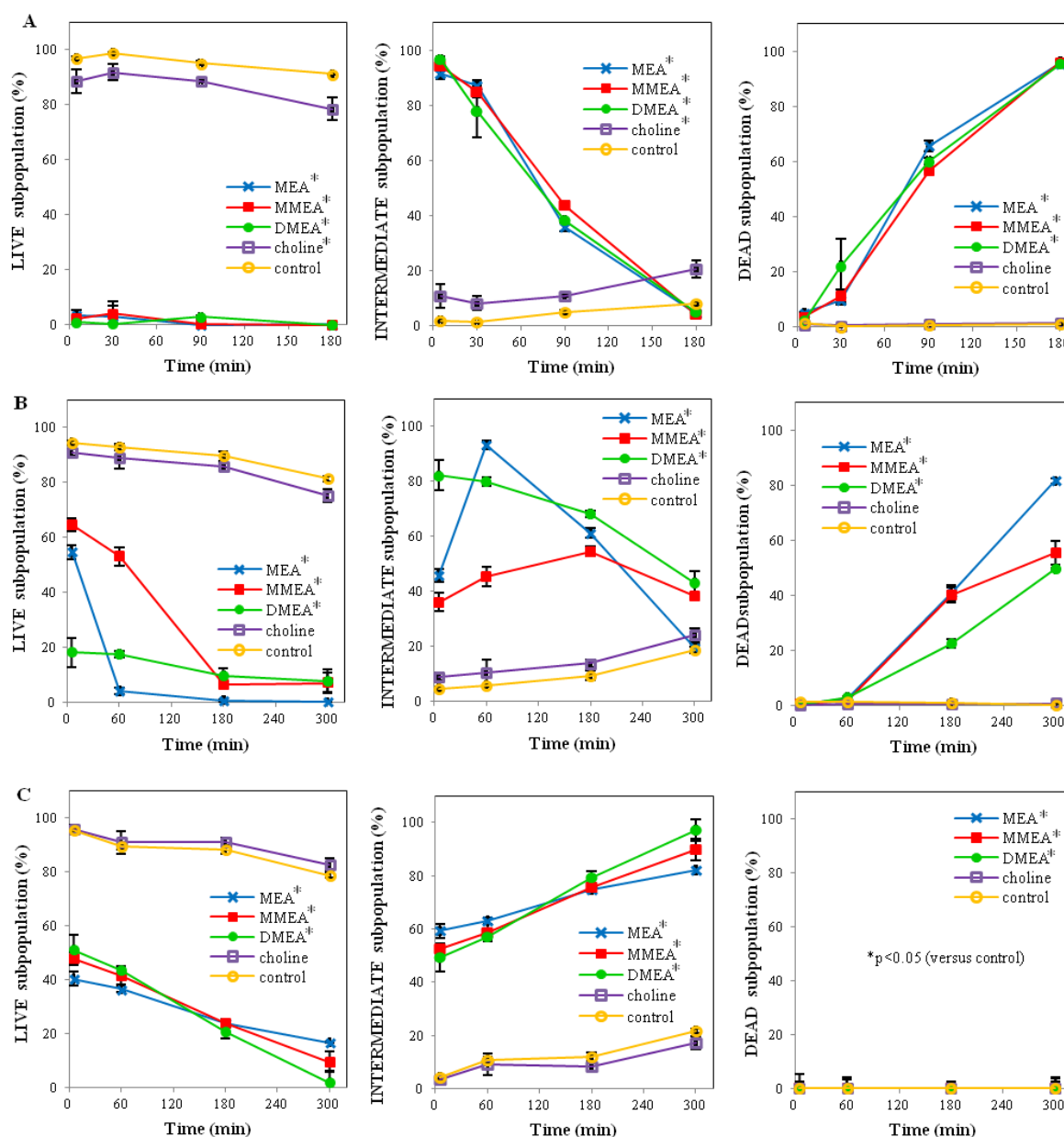


Figure 3: Percentage of membrane-intact or live, membrane-damaged or “intermediates” and irreversibly membrane-damaged or dead subpopulations of log phase *E. coli* (A), stationary phase *E. coli* (B) and log phase *P. oleovorans* (C). The bacterial populations were exposed to MEA, MMEA, DMEA, choline (50 mM, pH 9.5) and sterile PBS (control, pH 9.5). Data are expressed as means $\pm$ SD of triplicate experiments



ATP measurement

Total ATP levels were examined for up to maximally 120 min of exposure to each of the 4 test compounds (50 mM, pH 9.5) compared to the control. Mean ATP concentrations $\pm$ SD are presented in Fig. 4.

For log phase *E. coli* there was an instant (already after 5 min) ATP depletion during incubation with MEA, MMEA and DMEA compared to the control ($p=0.003$, $p=0.001$ and $p=0.002$, respectively). Moreover, this depletion persisted in the population exposed to DMEA, while the bacteria exposed to MEA and MMEA showed a transient effect with a significant increase in ATP after 30 min of incubation. In the MMEA samples this transient character even led to an ATP level significantly higher than that of the control sample ($p=0.002$) at 30 min. However, after 60 min of incubation ATP levels for MEA and MMEA exposed samples were again significantly lower than the control sample ($p<0.0005$ for both). Choline-exposed bacterial populations showed a significant but also transient decrease in ATP concentration and this only after 15 min of incubation ($p<0.0005$), returning to the level of the control sample at 60 min of incubation.

In the stationary *E. coli* population there was also an instant (already after 5 min) ATP depletion after exposure to MEA, MMEA and DMEA ($p<0.0005$ for all). However, all 3 test compounds induced recovery at 30 min with even a significant increase in ATP levels compared to the control sample ($p<0.0005$ for MEA and $p=0.001$ for MMEA and DMEA). The strongest effect was seen after DMEA exposure with a peak at 60 min of incubation. After 120 min the ATP level had again decreased to a level comparable to the control sample. Choline-exposed samples showed no significant difference in ATP levels compared to the control sample.

In marked contrast to *E. coli*, for *P. oleovorans* none of the compounds induced a decrease in ATP concentration. Unexpectedly, there was only a significant increase in ATP when exposed to MEA, MMEA and DMEA for 15 min ($p=0.017$, $p=0.003$ and $p=0.002$, respectively). The control and choline exposed samples show a more gradual time-dependent increase of the ATP concentration for up to 60 min of incubation. After 120 min of incubation the ATP concentration of all samples returned to start levels.

For *E. coli* again a difference was seen between log phase and stationary phase populations. The instant (already after 5 min) ATP depletions observed upon exposure to MEA, MMEA and DMEA is comparable to the effects seen when log phase *E. coli* where exposed to glycine, sarcosine and DMG under the same conditions (Vanhauteghem et al., 2013). However, only in the DMEA exposed population the ATP depletion persisted during the whole incubation, while the MEA and MMEA exposed populations showed a transient increase in ATP level.

These results on the level of cellular ATP concentration also show a difference between *E. coli* and *P. oleovorans* regarding the effect of MEA, MMEA and DMEA. Remarkably, the energy homeostasis of *P. oleovorans* is not negatively influenced by MEA, MMEA and DMEA. This is in agreement with



the prior suggestion that *Pseudomonas* is much more withstanding to stress situations (Arana et al., 2010). Pseudomonads are known for their metabolic versatility and their ability to metabolize an extensive number of substrates, even toxic organic compounds (Moore et al., 2006). Ebert et al. (2011) recently stated that *P. putida* is characterized by a remarkable metabolic robustness to perturbations of its cellular ATP metabolism. This makes them highly resistant against energetic stresses. They also described that *E. coli* are more sensitive than *P. putida* to the effects of a proton ionophore (which forces the bacteria to spend more ATP to compensate for H^+ influx). Of relevance, some *P. putida* subspecies were former classified as *P. oleovorans* species, possibly explaining why the *Pseudomonas* spp. used in the current study showed no ATP depletion upon exposure to the alkanolamines at alkaline pH, compared to *E. coli* (van Beilen et al., 2001).

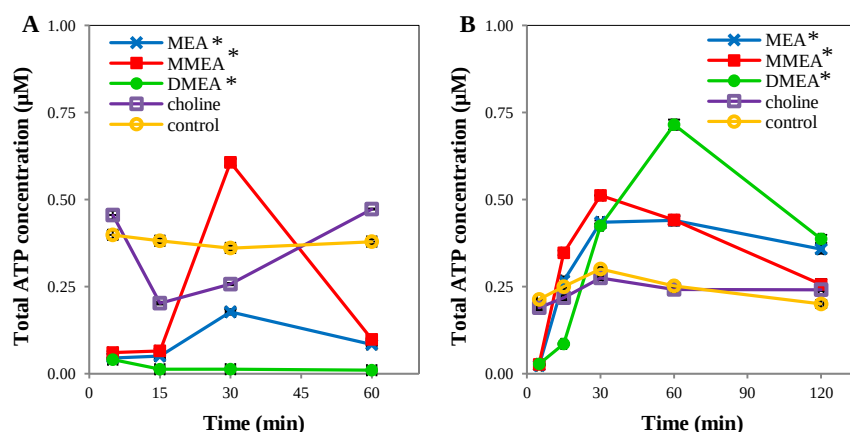


Figure 4: Total ATP concentrations of log phase *E. coli* (A), stationary phase *E. coli* (B) and log phase *P. oleovorans* (C). The bacterial populations were exposed to MEA, MMEA, DMEA, choline (50 mM, pH 9.5) and sterile PBS (control, pH 9.5). Data are expressed as means $\pm$ SD of triplicate experiments

CONCLUSION

In this study, we aimed to validate the hypothesis that intracellular proton scavenging can cause bacterial viability loss, as previously described for glycine, sarcosine and DMG on *E. coli* (Vanhautehem et al., 2013). The current data on the antibacterial effect of the three related alkanolamines MEA, MMEA and DMEA confirm our hypothesis for *E. coli*. Log phase *E. coli* show an immediate ATP depletion, followed by membrane permeabilization and loss of culturability, when exposed to glycine and its mono- and di-*N*-methylated derivatives, as well as to ethanolamine and its mono- and di-*N*-methylated derivatives under alkaline stress conditions. Stationary phase *E. coli* where more resistant, but as shown upon prolonged exposure to glycine, sarcosine and DMG at high pH, the bacterial viability is also lost (Chapter 2). Data on *P. oleovorans* on the other hand only partially confirm our hypothesis formulated for the mechanism for *E. coli* (Vanhautehem et al., 2013). Indeed, although the MIC data show a strong antibacterial effect of MEA, MMEA and DMEA over a wider range of pH values (pH 7.5-10.0) and both the culturability and membrane integrity



confirm these antibacterial properties (albeit not as strongly as seen for *E. coli*), the ATP data did not concur between both Gram negative bacterial species. We propose that a main factor responsible for the latter difference is the higher resilience of *Pseudomonas* species against stress conditions in general and alkaline pH stress in specific. Nevertheless, further research is clearly warranted to elucidate the underlying working mechanism of alkanolamines against pathogens from in-use MWF. Additionally, the advantages of non culture-based viability analysis should also be further exploited in metalworking fluid control systems.

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General Discussion



Exposure to glycine, sarcosine and DMG, but not betaine nor a saline control, induces viability loss of *E. coli* at alkaline pH. The information available to explain the underlying mechanism of this reproducible observation was however scarce. Therefore, the main of this PhD thesis was to indentify the direct or indirect antibacterial action of glycine and its methylated amine derivatives. This was achieved by focusing on the assessment of a combination of cellular targets that could be affected by these specific compounds, and which are essential in bacterial metabolism.

Briefly, we determined a clear loss of *E. coli* membrane integrity, preceded by an instant depletion of the bacterial cellular energy and hyperpolarization of its cytoplasmic membrane. This antibacterial effect was confirmed by traditional viability indicators, such as the decrease in culturability and the establishment of MIC values at alkaline pH.

Several questions on the proposed antibacterial effect and underlying mechanism of this effect, of glycine and its *N*-methylated derivatives sarcosine and DMG (but not betaine) can now be answered.

1 The added value of viability analysis

Viability assessment of microorganisms is relevant for a wide variety of applications ranging from fundamental microbiology to industrial applications. It assists in the evaluation of inactivation treatments, detection and quantification of food spoilage microorganisms, quality assessment of starter cultures, and many others (Breeuwer and Abee, 2000). The question whether a bacterial cell is active or inactive remains however a continuous cause of discussion. Also, heterogeneity is a specific hallmark of microbial systems (Rezaeinejad and Ivanov, 2011). Bacterial cultures have a remarkable capacity to display different metabolic levels and vital stages in one culture (Lethinen et al., 2004). So as a first step, the determination of viability categories of bacterial cells must be made. There are at least 3 categories that should be considered: (i) actively growing cells; (ii) living but inactive cells, which still have potential activity, often defined as “dormant” and referred to as “intermediate” in this PhD thesis; (iii) dead inactive cells (Lebaron et al., 2001; Sträuber and Müller, 2010).

Dormancy or “intermediate” is a state in relation with both live and dead cells, but its current definition is very diverse. It is often defined as a possibly reversible state with a very low level metabolic activity and the absence of culturability (Oliver, 2005). However, different grades can be assigned to these intermediates, all reflecting different levels of activity and culturability. Our results confirmed the existence of such heterogeneity of bacterial subpopulations. Analysis with the LIVE/DEAD BacLight™ kit indeed revealed a wide array of intermediate bacteria, showing different intensities of staining with PI and SYTO9, and correspondingly different levels of culturability. It is therefore obvious that the dormant or intermediate bacterial populations can consist of both cells that have ceased growth due to injury, as well as viable but non-culturable (VBNC, see [General](#)



Introduction) bacteria. There is a marked distinction to be made between both these groups. On the one hand, the injured state can be either transient or lead to cell death and is usually the consequence of cumulative cellular damage. The shift to the dead phase occurs when the extent of the injury is beyond repair and growth cannot be resumed. On the other hand the transition to the VBNC state is usually the consequence of a specific process to ensure long-term survival in suboptimal conditions (Oliver, 2005).

The traditional culture-based methods are essential in the determination of viability, but not sufficient as a sole parameter as they provide no real-time information (Hammes et al., 2011). They only yield results on a total population level, while the true viability remains unclear. Currently a wide range of methods has become available to study microorganisms at a greater level of detail. Numerous fluorescent dyes have been suggested to act as viability probes because they can detect changes in the physiology or metabolism of the cells (Mortimer et al., 2000; Hammes et al., 2011). It is thus crucial to choose a method that relates closely to the expected effects and to combine several viability parameters in order to correctly interpret the gathered data and come to a valid conclusion on bacterial viability as highlighted in the General Introduction (Sträuber and Müller, 2010; Hammes et al., 2011; Fig. 1).

2 The antibacterial effect described for glycine

Glycine is the most simple amino acid structure, and it is a non-essential amino acid for bacteria (Pizer, 1965). Nevertheless, membrane permeabilizing characteristics have been attributed to this small organic molecule by several authors (Dempsey, 1973; Hammes et al., 1973; Schwartz et al., 1979; Ikura, 1986; De Jonge et al., 1996; Hu et al., 2002; Minami et al., 2004; Li et al., 2010). Moreover, high concentrations of glycine induce bacteriolysis or morphological alterations in various bacterial species, including *E. coli* (Ikura et al., 1986). As stated in the General Introduction (section 3.1), the mechanisms of this antibacterial effect of glycine is based on a defective peptidoglycan structure resulting in a permeabilization of the bacterial cell wall structure (Hammes et al., 1973). All previous experiments referenced above were performed under physiological conditions with varying glycine concentrations. Unexpectedly, we did not observe such an effect at physiological pH in our experiments, and only could show an antibacterial effect of glycine under alkaline conditions. In most of the previous experiments however, both the glycine concentration as well as the medium composition differed substantially from our incubation conditions. More importantly, reported incubation times were at least 24 hours, while we typically tested more rapid effects (i.e. between 5 min and 6 h) on bacterial viability and not after prolonged (> 24 h) incubation. Finally, the antibacterial effect of glycine is based on a disturbance of peptidoglycan synthesis during bacterial



growth. Our viability analyses were performed under conditions of bacterial survival, in physiological saline, where no bacterial growth is to be expected. All these provided arguments may well explain why we did not see an antibacterial effect of glycine at physiological pH in our experiments.

Another important remark is that the antimicrobial mechanism (alanine substitution) described for glycine in the [General Introduction](#) certainly cannot be expected for sarcosine or for DMG because the latter two *N*-methylated derivatives are not able to form the typical peptide bond involved in the replacement of D-alanine by glycine, responsible for the formation of a defective peptidoglycan structure.

3 The emulsifying characteristics attributed to *N,N*-dimethylglycine (DMG) and betaine

Research in the context of animal nutrition performed by Kalmar et al. (2010a,b) and Cools et al. (2010) led to the assumption that DMG might possess emulsifying characteristics. These authors describe beneficial effects of DMG on nutrient digestibility both in broilers (Kalmar et al., 2010a,b) and pigs (Cools et al., 2010). As for betaine, much research in the context of animal (pig) nutrition was done by Eklund et al. (2005 and 2006a,b) stating an improved fat digestibility due to dietary betaine supplementation.

In [Chapter 1](#) of this PhD thesis we therefore investigated the emulsifying/surfactant potential of glycine, sarcosine, DMG and betaine, describing the emulsifying stability index (ESI) for all compounds under both physiological/neutral and alkaline pH conditions. Data obtained under acidic pH conditions were present (Fig. 2) in this first experimental data chapter. Results for all evaluated pH values show that only DMG is a potentially mild enhancer of emulsification, independent of the pH applied. We also tested the influence of the four compounds on the cytoplasmic membrane lysis of red blood cells ([Chapter 1](#) - Table 3). This latter parameter is used to demonstrate the hemolytic properties of compounds with surfactant characteristics, as an indicator for cytotoxicity (Rinaldi et al., 2002). Because the mammalian erythrocyte has no internal organelles, it is a very common cell membrane system to study molecule-membrane interactions based on an emulsification related destruction of membrane phospholipids (Svetina et al., 2004; Martinez et al., 2007). Results showed no significant effect of either of the four compounds, nor under acidic, physiological/neutral or alkaline pH conditions.

These findings were unexpected. We had presumed that it was quite conceivable for these compounds to have emulsifying potential for several reasons. First, as mentioned in the last part of the [General Introduction](#) (Section 3 – Fig. 3), a distinction has to be made between betaine on the one hand, and glycine, sarcosine and DMG on the other hand. Indeed, betaine is a zwitterion, retaining its opposite charges over the pH range of pH 6.5 to pH 10.0, i.e. a positively charged amine group and a negatively



charged carboxylic acid group. Glycine, sarcosine and DMG can be classified as amphoteric, which implies that depending on the pH, they can either become anionic, zwitterionic/neutral or cationic. Both amphoteric and zwitterionic characteristics can result in mild emulsifying actions (Zajic and Panchal, 1976).

Literature provides several examples of molecules, closely related to our compounds with surfactant traits. Clapés and Infante (2002) describe bio-based surfactants, including amino-acid derived molecules that, due to their amphoteric nature, have emulsifying properties. Based on their pK_a values, glycine, sarcosine and DMG are all zwitterionic at physiological pH, but an increasing fraction of the molecules will be anionic at alkaline pH. Anionic surfactants are known as detergents, emulsifiers, wetting and antistatic agents, and lubricants (Mohamed and Mohamed, 2004; Badawi et al., 2007). However, common in the large majority of amino acid-based surfactants is the need for a hydrophobic tail, typically presented as *N*-alkyl derivatives of simple amino acids. The hydrophobic nature of these surfactant tails allows them to interact with and disrupt cytoplasmic membranes (Sánchez et al., 2007). The compounds described in this thesis do not possess such a hydrophobic tail and they only change their ionization state with varying pH. The lack of such a distinct hydrophobic part could explain why we were not able to find proof for a significant emulsifying action for glycine, sarcosine, DMG and betaine.

Nevertheless, our ESI data indicate that DMG has a mild emulsifying potential compared to glycine, sarcosine, betaine and the control sample. This could be explained by its possible hydrotropic characteristics. Hydrotropy refers to the ability of highly water-soluble but mildly surface-active amphiphilic organic molecules (so-called “hydrotropes”) to increase the emulsification of poor soluble or water-insoluble organic compounds in aqueous solutions (Roy and Moulik, 2002). Hydrotropes usually have relative short hydrocarbon chains or hydrophobic groups. It is suggested that they can increase the permeability of a cellular membrane by inducing a change in the molecular organization of the cell membrane (Raman and Gaikar, 2002).

4 The *E. coli* membrane altering effects of glycine, sarcosine and DMG under alkaline stress

Although the ESI and red blood cell hemolysis data did not indicate any direct effect of the four compounds, a membrane damaging effect on *E. coli* was found for glycine, sarcosine and DMG under alkaline conditions with LIVE/DEAD BacLight™ flow cytometry, as described in [Chapter 2](#). This increase in membrane permeability occurred only under alkaline stress and not with physiological/neutral, nor more acidic (pH 6.5) pH conditions. There was also no association between the degree of membrane damage and the compound-specific pK_a or degree of methylation. This



observation raised the fundamental questions whether the pH-induced alteration in ionization state of glycine, sarcosine and DMG enables them to cause direct membrane damage, or whether the alkaline pH first affects bacterial homeostasis mechanisms, allowing the latter compounds to affect *E. coli* viability through structural damage, or whether it is a combination of both effects?

4.1 The effect of alkaline conditions on *E. coli*

Under normal conditions, the cytoplasmic pH is buffered by small organic molecules such as amino acids, as well as by ionizable groups from proteins and inorganic polymers such as polyphosphate (Slonczewski et al., 1982; Slonczewski et al., 2009; Krulwich et al., 2011). Despite these various sources of buffering, there is still a strong need for active mechanisms of pH homeostasis (Slonczewski et al., 2009). Based on the growth permitting pH range, bacteria can be divided in 3 classes: acidophilic (pH 1-3), alkaliphilic (pH 10-13) and neutralophilic (pH 3-10, e.g. *E. coli*) (Krulwich et al., 2011). Ionophores, permeant acids or bases can cause a shift of the cytoplasmic pH by influx or efflux of protons, emphasizing the need for active compensation mechanisms.

In the case of membrane permeant bases, an external alkaline pH will favor their intracellular accumulation. Under these conditions, such dissociated bases will enter the cell and be protonated in the cytoplasm. This consumption of cytoplasmic protons can impair pH homeostasis as it puts a strain on the already energetically stressed bacteria (Repaske and Adler, 1981; Yohannes et al., 2005). The latter phenomenon is illustrated by the toxic effect of high organic amine concentrations on alkaliphilic bacteria. They are at risk of accumulating ammonium at the expense of cytoplasmic protons, thereby comprising their pH homeostasis mechanisms (Wei et al., 2003). Wei et al. (2006) also investigated the importance of active pH homeostasis for neutralophilic bacteria, suggesting that ammonium could be toxic for neutralophiles under alkaline conditions due to a disturbance of active pH homeostasis mechanisms.

For *E. coli*, numerous responses to pH stress are known and the mechanism by which these bacteria maintain their internal pH near 7.6 is very complex but extensively studied (Maurer et al., 2005; Slonczewski et al., 2009; Wilks et al., 2009; Krulwich et al., 2011). Under alkaline stress, homeostasis strategies enclose both active and passive adaptations of the bacterial cells' physiology, which are all involved in the effort to acidify the cytoplasm, as well as general stress responses. Active strategies first involve the direct uptake of protons by use of a variety of transport mechanisms. Several membrane bound systems are upregulated to generate as many intracellular protons as possible (Padan et al., 2005). It is generally accepted that the bacterial cytoplasmic pH is regulated by both the H⁺ translocating ATP synthase, as well as various cation transport systems (Nyanga-koumou et al., 2011), as illustrated in Fig. 1. Generation of a substantial $\Delta\Psi$ to maintain an adequate proton motive force (PMF) at high pH is crucial to support the central role of monovalent cation/proton antiporters in



alkaline pH homeostasis in respiratory bacteria (Padan et al., 2005; Krulwich et al., 2011). Most free-living bacteria have multiple Na^+/H^+ and K^+/H^+ antiporters that each contribute to pH, cationic and osmotic homeostasis (Padan et al., 2004). Antiporters with roles in alkaline pH homeostasis catalyze electrogenic antiport in which the ratio of H^+ influx versus Na^+ or K^+ efflux is unequal (Macnab and Castle, 1987). Potassium transport also plays a role in pH homeostasis, possibly by storing energy to drive H^+ efflux or influx, either by symport or antiport mechanisms (Kitko et al., 2010). The issue of why cells have so many antiporters with overlapping specifications is an interesting one. It seems that the answer may be that they each have an added value at slightly different pH and ion concentration ranges, as well as having different and sometimes multiple monovalent cation profiles (Radchenko et al., 2006).

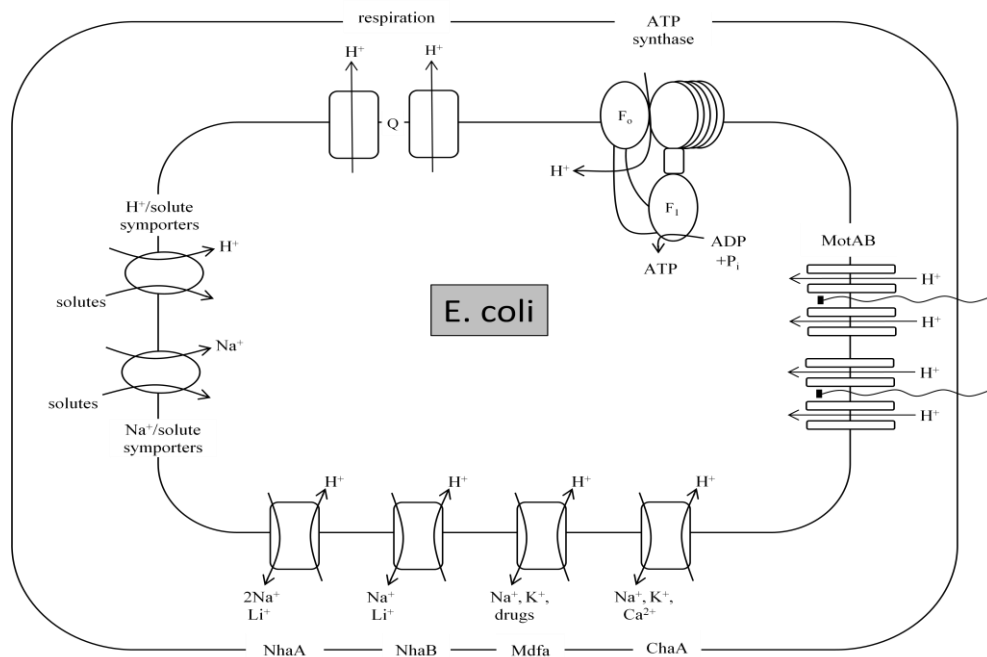


Figure 1: Role of membrane-bound proton transport systems in *E. coli* pH homeostasis.

ATP synthase imports H^+ during ATP synthesis. NhaA exchanges 2H^+ for 1Na^+ , enabling proton entry driven by the $\Delta\Psi$ component of the PMF (Arkin et al., 2007). The intracellular level of K^+ in *E. coli* is regulated through various transport systems. Lewinson et al. (2004) described that MdfA, a multidrug transporter, participates in the regulation of *E. coli* pH homeostasis at external alkaline pH (up to pH 10) by Na^+/H^+ and K^+/H^+ exchange. Radchenko et al. (2006) report on the K^+/H^+ exchange activity of ChaA, which was previously known to regulate $\text{Na}^+, \text{Ca}^{2+}/\text{H}^+$ antiport activity in *E. coli*. ChaA extrudes K^+ against both outwardly and inwardly directed K^+ concentration gradients. The K^+/H^+ activity of ChaA is also pH-dependent, with an onset at pH 9.0. Next to these membrane porters, there is also evidence for the role of several other ion pumping systems aiding in the pH homeostasis of *E. coli*, such as NhaB, $\text{Na}^+, \text{H}^+/\text{solute symporters}$ and the flagellar motor MotAB (Adapted from Padan et al., 2005; Krulwich et al., 2011)



A second active strategy of maintaining pH homeostasis under alkaline conditions is the remodeling of various metabolic patterns. This implies both a regulation of H^+ consumption and the generation of extra H^+ or other acidic compounds by metabolic enzymes. Bacteria direct overall amino acid catabolism into pathways that remove ammonia and generate acids. This includes deaminases which provide an acid-generating mechanism that is adaptive to alkaline challenge. Especially the amino acids tryptophan and serine (but also cysteine) are deaminated, as illustrated by the upregulation of TnaA (tryptophan deaminase), TnaB (a tryptophan transporter) and SdaA (serine deaminase) during alkaline stress (Blankenhorn et al., 1999; Stancik et al., 2002; Bordi et al., 2003; Maurer et al., 2005; Wei et al., 2006). Other enzymes that channel metabolites towards fermentation acids instead of excreting amines are also upregulated (Stancik et al., 2002).

In addition to these active strategies, passive support is generated by a change in membrane H^+ permeability and other cell surface changes allowing a better capitation and surface retention of protons (Clejan et al., 1986; Stancik et al., 2002; Slonczewski et al., 2009; Mesbah et al., 2009). These are mechanisms also seen in alkaliphilics, for example Mrp proteins which are used to funnel protons into antiporters in extremely alkaline media (Swartz et al., 2005; Morino et al., 2008). Some sort of sequestration of H^+ is widely thought to be used for proton-coupled alkaliphilic oxidative phosphorylation, but the exact mechanism is still to be defined (Slonczewski et al., 2009).

Exposure to alkaline environments also triggers several general stress responses. The SOS response is induced in neutrophilic *E. coli* upon cytoplasmic alkalinization (Schuldiner et al., 1986). An alkaline shift of the external pH, but not the cytoplasmic pH, induces heat shock proteins DnaK and GroE (Taglicht et al., 1987; Sampethkumar et al., 2004; Sharma and Beuchat, 2004). Small et al. (1994) report the requirement of RpoS for survival of *E. coli* when exposed to pH 9.8. RpoS is a central regulator during the response to many stress conditions (Jozefczuk et al., 2010; Battesti et al., 2011). Of relevance, ATP levels directly control RpoS stability (Peterson et al., 2012). Moreover, several stress responses interact with pH stress, including oxidative stress, heat shock, envelope and osmotic stress (Maurer et al., 2005; Wilks et al., 2009; Kitko et al., 2010). Alkaline pH also induces the Cpx signaling system that monitors the cell envelope in *E. coli*, upregulating genes that encode factors involved in envelope maintenance and extracytoplasmatic chaperones and proteases (Ruiz and Silhavy, 2005; Sikdar et al., 2012).



4.2 The described effects of glycine and its *N*-methylated derivatives on *E. coli* membrane transporters

Glycine, sarcosine, DMG and betaine have been described in relation to osmotic stress in several bacterial species. Especially the role of betaine as an osmoprotectant for bacteria under osmotic stress was well studied in the past decade (Culham et al., 2001; Ly et al., 2004; Tøndervik and Strøm, 2007). In contrast, the potential role of these compounds under pH stress conditions has never been thoroughly investigated. Nevertheless, evidence is available which allows us to propose an overlap between osmotic and pH stress in bacteria.

Osmotic stress causes a transient alkalization of the cytoplasm due to the expulsion of H^+ to compensate for the uptake of K^+ (Csonka, 1989). Interplay between changes in osmolarity and pH occurs because some monovalent cation/ H^+ antiporters have roles in both osmo-adaptation and pH homeostasis (Padan et al., 2005). Additionally, an overlap between alkaline and salt stress can be deduced from the significant increase in Na^+ toxicity when the pH rises (Small et al., 1994; Padan et al., 2005). Kitko et al. (2010) also suggest a link between osmolytes and (acid) pH stress. Although these authors could not clarify the mechanism of the observed link between osmoregulation and pH homeostasis, they state that cell volume changes and stabilizing ion fluxes might be involved. More specifically, they describe a positive effect of osmolytes like choline chloride and proline on pH homeostasis under acidic stress conditions in *E. coli*.

The role of glycine and its derivatives under pH stress conditions is not well documented for neutrophilic bacteria such as *E. coli*. Giotis et al. (2010) investigated gene expression in *Listeria monocytogenes* under alkaline stress conditions and showed an upregulation of osmolyte transporter systems responsible for the uptake and accumulation of potent osmolytes such as carnitine, choline and betaine. Interestingly, betaine and its transporters have been investigated in alkalophilic bacteria where a high transport activity was seen, particularly at alkaline pH (Laloknam et al., 2006; Bhargava et al., 2011). Laloknam et al. (2006) also investigated a potential inhibitory effect of glycine, sarcosine and DMG on the uptake of betaine, but no negative effect of these three compounds was observed. Alkaline pH stress in *Listeria monocytogenes* was found to induce gene expression, more specifically an upregulation of systems that are normally induced under other types of stresses, e.g. osmotic stress (Giotis et al., 2010). Two explanations were proposed for these effects: (i) osmolytes have been described to exert broad stabilizing effects, e.g. carnitine, glycine, sarcosine, DMG and betaine have been shown to maintain the cells' protein configuration (Singh et al., 2004); (ii) toxic levels of Na^+ , originating from the strong base NaOH, could be responsible for the induction of Na^+ /solute symporters or antiporters.

Evidence on how glycine, sarcosine and DMG could negatively influence membrane transporters involved in bacterial pH homeostasis was not provided nor by literature nor by the data of this PhD



thesis. Many membrane transport systems are involved in maintaining homeostasis (Fig. 1). Yet, to date it is not clear how certain stress factors and the systems induced by them, are interconnected. Interestingly, a competitive inhibition of the osmotically induced membrane transporter ProP was described between betaine on the one hand and sarcosine and DMG on the other hand (MacMillan et al., 1999). Nevertheless, it is not known if and how this inhibition is related to the effects described in this PhD thesis. Further research is therefore warranted into the mechanism by which glycine and its derivatives affect specific bacterial membrane transporters.

5 The effect of pH on glycine and its *N*-methylated derivatives

As stated above, many antimicrobial compounds are weak acids or weak bases. For both types of molecules, the external pH determines their degree of protonation and subsequently their ionization state. This principle underlies the antibacterial potential of these compounds.

Organic acids provide a typical and well-studied example of how molecules exert an antibacterial effect through a change in the degree of protonation caused by a varying environmental pH (Booth, 1985; Roe et al., 2002; Lu et al., 2011). The antibacterial effect of organic acids is based on this principle and consists of several components as demonstrated in Fig 2. In their non-dissociated and thus neutral form they are able to freely diffuse through lipid bilayers, or pass through specific transporters (Lu et al., 2011) and then deprotonate in the more alkaline cytoplasm, decreasing the cytoplasmic pH (Booth, 1985). These non-dissociated molecules also directly interact with the lipid bilayer at low extracellular pH (Stratford and Anslow, 1998; Roe et al., 2002). The intracellular proton release has several consequences: anion accumulation, leading to osmotic stress, and disturbance of pH homeostasis (Roe et al., 2002; Van Immerseel et al., 2006). In an acidic environment, organic acids are non-dissociated. When entering a microbial cell, the pH rises and, depending on its specific pK_a -value, the organic acid dissociates by releasing a proton into the cytosol. This process forces the microbial cell to spend more energy to extrude protons in order to maintain pH homeostasis. Decrease in intracellular pH will disrupt enzymatic reactions and many other cellular functions (Dibner and Buttin, 2002). Lu et al. (2011) additionally suggest that other factors, next to cytoplasm acidification, should be taken into account. A weak acid may have multiple cellular targets, including membrane integrity, biosynthesis of proteins and nucleic acids, activity of critical enzymes and osmotic homeostasis. More than a decade ago, Roe et al. (2002) already postulated such effects by describing the inhibitory effects of acetate on the methionine biosynthetic pathway.

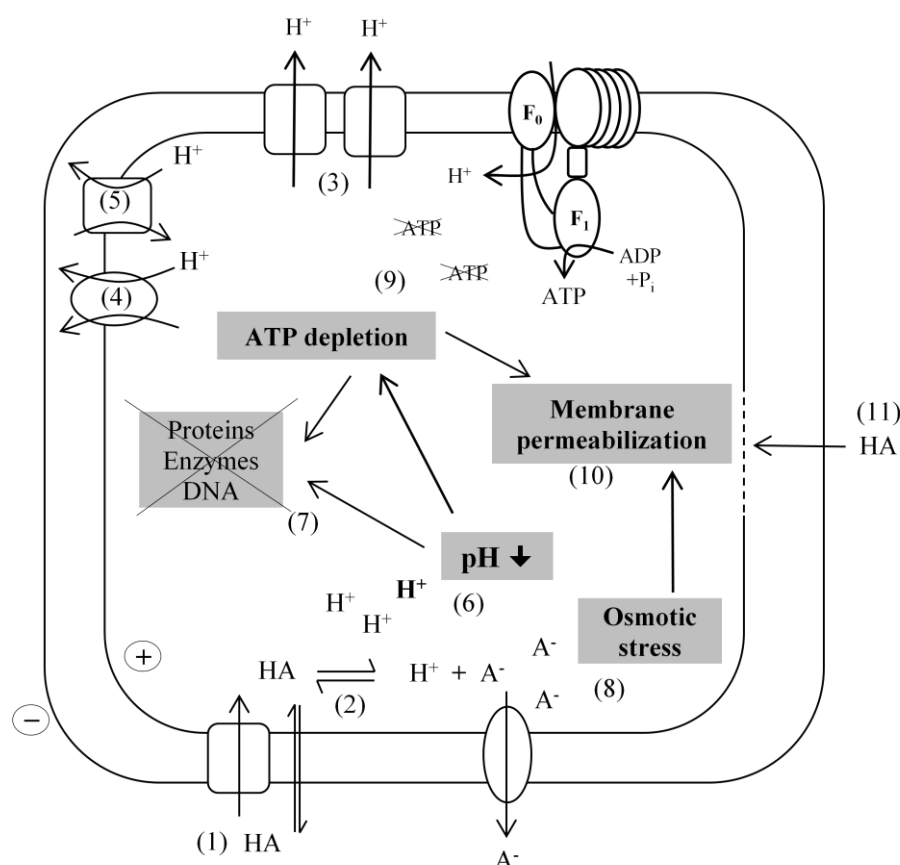


Figure 2: The described antibacterial effect of weak organic acids.

Non-dissociated organic acids (HAs) enter the bacteria by passive diffusion or through membrane transport systems (1). Once intracellular, they dissociate (2) liberating protons and anions into the cytoplasm. Protons are extruded during respiration (3) and by proton symporters (4) and antiporters (5). A cytoplasmic proton excess will lead to a decrease of the pH_i (6), which has severe consequences for protein and DNA synthesis, as well as enzyme activities (7). In addition, anion accumulation is toxic and leads to osmotic stress (8). Both the pH and osmotic homeostasis require the cell to spend extra energy, i.e. ATP, leading to a progressive depletion of the ATP pool (9) and eventual disruption of cellular metabolism and loss of membrane integrity (10). Some organic acids are also able to intercalate into the lipid bilayer at low pH (11)

Of relevance for this PhD thesis, the consumption of energy, i.e. ATP, in an attempt to keep intracellular pH within physiological range can thus lead to a loss of bacterial viability. As demonstrated for organic acids, such transport of protons consumes energy, draining the cell of ATP and possibly leading to cell death (Hosein et al., 2011). In [Chapter 2](#) we measured the ATP concentration of *E. coli* populations exposed to glycine, sarcosine, DMG and betaine at pH 9.5. As stated before, glycine, sarcosine and DMG (but not betaine) can be considered as weak bases as they all possess an amine group that can be protonated. In [Chapter 2](#) we suggest that a fraction of the glycine, sarcosine and DMG (but not betaine) molecules have a deprotonated amine group at pH 9.5, the extracellular pH stress used in our experiments. As these three compounds go intracellular, they



become protonated in the cytosol, which is buffered at physiological pH. This proton scavenging effect is very intriguing and we hypothesize that it is responsible for the instant depletion of the ATP pool in our bacterial population because the cell needs to spend extra energy to generate/import protons and to prevent intracellular alkalization. This amplifies the pH stress and is likely the trigger for a cascade of other cellular dysfunctions.

This principle of intracellular proton scavenging by basic compounds was previously described by a few other groups, albeit in a different context. Yohannes et al. (2005) evaluated the role of alkaline pH in polyamine stress. These authors postulated that the intracellular accumulation of polyamines is favored when the cytoplasmic pH is lower than the external pH. Under such conditions, the uncharged base entering the cell is protonated in the cytoplasm and its consumption of protons can impair pH homeostasis. Booth (1985) already described the addition of diethanolamine, of which the amine group can act as a proton-acceptor, to raise the intracellular pH of a cell suspension. Taglicht et al. (1987) not only confirmed this effect and described the mechanism, but they also state that protein synthesis is not affected by the addition of up to 20 mM of diethanolamine. Finally, weak bases e.g. methylamine and related amines, have been used to measure the intracellular pH. Again, these deprotonated probes will cross the membrane and once intracellular become protonated providing a read-out for the cytoplasmic pH (Slonczewski et al., 2009; Krulwich et al., 2011).

6 How ATP depletion can lead to membrane permeabilization

In [Chapters 1 and 2](#) we described the clear loss of *E. coli* membrane integrity due to exposure to glycine, sarcosine and DMG under alkaline conditions. This effect occurs after 3 to 6h of exposure in stationary bacteria and already after 30 min in exponential phase bacteria. In [Chapter 2](#) we additionally investigated the ATP concentration of those bacterial populations, and observed an instant ATP depletion i.e. after 5 min of incubation. By further investigating the effects on selected cellular targets, we aimed to at least partially elucidate the causal relationship between both these processes.

As the ATP concentration is closely related to bacterial respiration we flow cytometrically measured the *E. coli* respiration rate. When bacterial cells are exposed to alkaline stress, there is a repression of proton extruding respiratory chain complexes (Maurer et al., 2005; Krulwich et al., 2011). In line with the latter, our results show a minor and transient, but significant decrease in respiratory activity when the bacteria are exposed to glycine and sarcosine for 15 min. There is no further indication obtained within this PhD thesis that the bacterial respiratory function is directly affected. With further respect to the proton homeostasis, Hayes et al. (2006) report the upregulation of several genes in the ATP



synthase operon of *E. coli* under alkaline stress. Krulwich et al. (2011) confirm that under these conditions, requiring proton influx, *E. coli* elevates the expression of the proton pumping F_0F_1 -ATP synthase. This increase in number of ATP synthase complexes increases the H^+ influx and compensates for a decreased PMF (required for efficient ATP synthesis) at high pH.

A parameter closely related to both ATP production and membrane integrity in *E. coli* is the membrane potential ($\Delta\Psi$). For all bacteria, maintenance of their $\Delta\Psi$ is essential for survival (Hammes et al., 2011). It represents a voltage difference between the inside and outside of a cell generated by the translocation of protons by the electron transport chain. Together with the pH gradient across the bacterial cytoplasmic membrane (ΔpH) it produces the proton motive force (PMF) (Dimroth and Cook, 2004). The $\Delta\Psi$ is essential for (i) ATP synthesis; (ii) active membrane transport systems; (iii) motility (flagella); and (iv) maintenance of the intracellular pH (Breeuwer and Abee, 2004; Bot and Prodan, 2010). Our data in [Chapter 2](#) show a hyperpolarization of *E. coli* due to exposure to glycine, sarcosine and DMG at pH 9.5. This was unexpected as membrane depolarization is most commonly associated with membrane permeabilization. However, other authors also describe hyperpolarization prior to membrane integrity loss and decreased viability. Indeed, Spindler et al. (2011) recently described the effects of the antimicrobial peptide Bac8c, which disrupts normal electron flow and causes membrane hyperpolarization. These authors state that the latter process then leads to the formation of superoxide radicals, which have been widely implicated in bacterial killing (Kohanski et al., 2007). Prior to this study, Sánchez et al. (2010) examined the antibacterial potential of edible and medicinal plants and had also observed these compounds to cause hyperpolarization, rather than the expected depolarization. They state that this effect is due to a shift of the intracellular pH affecting cellular homeostasis based on results presented by Bot and Prodan (2010). Membrane permeabilization preceded by hyperpolarization was also reported in a third study by Yount et al. (2009) as an effect of a specific antimicrobial peptide on *E. coli*.

Based on the data gathered with the determination of a complementary set of viability parameters (as outlined in the [General Introduction](#), Fig. 1) and on in-depth literature research, we can now propose a hypothesis on the mechanism underlying the antibacterial effect of glycine and its mono- and dimethylated *N*-derivatives, sarcosine and DMG under alkaline stress conditions. As these three unprotonated compounds enter the neutral cytosol under alkaline extracellular conditions, they immediately become protonated. This H^+ scavenging causes both a hyperpolarization of the cytoplasmic membrane (more negative intracellular, Fig. 3) and a higher ATP consumption in an effort to sustain pH homeostasis. The combined hyperpolarization and energy depletion induce loss of membrane integrity, which is to a limit reversible upon restoration of a physiological pH. However, if the alkaline exposure to these compounds is prolonged, the combined stress will finally lead to



bacterial cell death. We were able to establish this concept for *E. coli*, providing opportunities to validate such an antibacterial mode of action for other bacterial species.

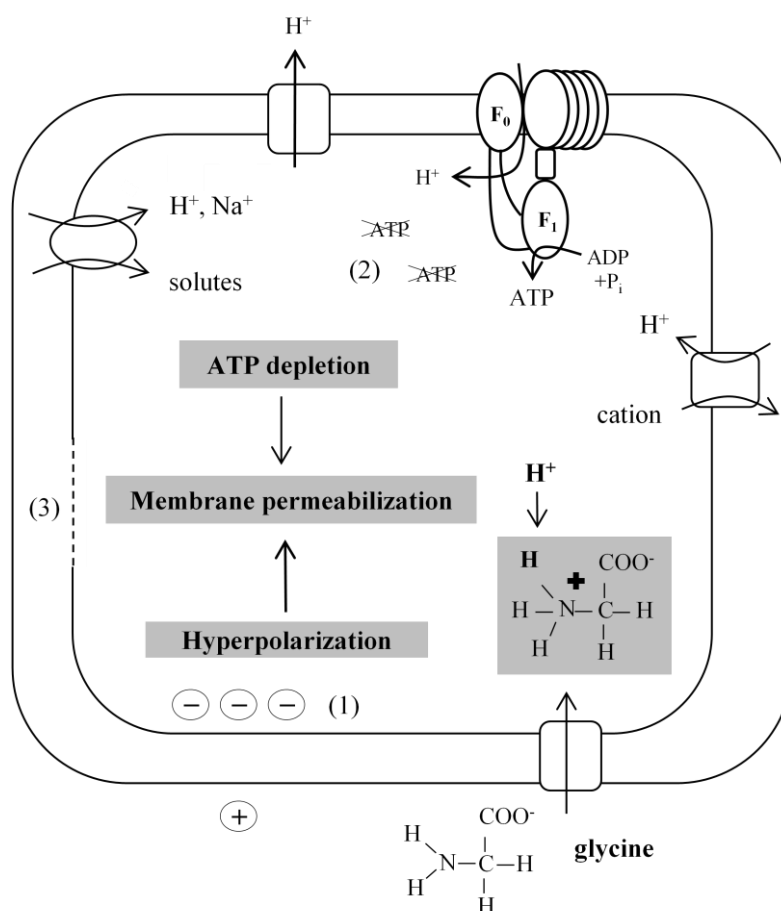


Figure 3: The proposed mechanism for the antibacterial effect of glycine, sarcosine and DMG at alkaline pH.

When unprotonated glycine enters the neutral cytosol under extracellular alkaline conditions it becomes protonated. This causes membrane hyperpolarization (1) by proton scavenging and a higher ATP consumption in an effort to sustain pH homeostasis (2). These effects lead to a loss of membrane integrity (3)

7 A related compound series – is the hypothesis confirmed?

In order to validate the proposed concept of bacterial viability loss caused by intracellular proton scavenging ([Chapter 2](#)), we tested a second compound series i.e. that of ethanolamines. Monoethanolamine (MEA) is the simplest ethanolamine, used by bacteria as a carbon and/or nitrogen source and abundantly present as a part of the bacterial cytoplasmic membrane in the form of phosphatidylethanolamine (Garsin, 2010). In analogy with the glycine series of compounds, we also tested the effect of the mono-, di- and tri-methylated derivatives of MEA, respectively monomethylethanolamine (MMEA), dimethylethanolamine (DMEA) and choline at alkaline pH. The



three test compounds MEA, MMEA and DMEA all have an amine group capable of binding H^+ under physiological conditions. The structure and pK_a values of these compounds ([Chapter 3](#), Table 1) confirm that a fraction of these molecules (depending on both pK_a and environmental pH) will be present as neutral molecules at pH 9.5, able of scavenging protons upon entry in the neutral bacterial cytosol. In marked contrast, choline is, like its analogue betaine, not able to bind an extra H^+ , and was therefore not expected to cause bacterial viability loss.

Several decades ago, Bennett et al. (1979) already reported on the antimicrobial properties of a variety of ethanolamines. They state that propanolamines, butanolamines and ethanolamines all exhibit significant inhibitory properties. The exposure to these alcohol-amines has been reported to impact bacterial growth through a variety of mechanisms including membrane damage, ion leakage, disturbance of pH homeostasis and the induction of several stress responses (Bowles and Ellefson, 1985; Rutherford et al., 2010). More specifically, the exposure of *E. coli* to n-butanol leads to a combination of stress responses, including cell envelope stress, oxidative stress (increased intracellular ROS levels), perturbation of respiratory systems, protein misfolding, acid stress and induction of efflux systems (Rutherford et al., 2010).

Ten years later, but still more than 2 decades ago, Sandin et al. (1990, 1991 and 1992) already showed that alkanolamines have an antibacterial effect, which is greatly enhanced at alkaline pH. This remarkable observation allowed them to conclude that the antimicrobial activity of these compounds is closely related to their uncharged form which occurs at this high pH. These latter authors also suggested that the underlying working mechanism could be due to the uncharged forms being able to easily penetrate the cytoplasmic membrane and subsequently becoming protonated within the cell (Sandin et al., 1990). More recently, Bakalova et al. (2008) specifically tested the microbial toxicity of MEA and again found a close relation between antimicrobial activity and pH. Finally, we previously proposed an antibacterial effect for glycine and its *N*-methylated derivatives, based on intracellular proton scavenging under alkaline pH stress ([Chapter 2](#); Vanhautehem et al., 2013).

Data on log phase *P. oleovorans* only partially confirmed the proposed working mechanism for this bacterial species. Although we were able to establish a clear antibacterial effect of MEA, MMEA and DMEA against *P. oleovorans*, there was no depletion of the ATP pool and membrane permeabilization appeared less severe for this concentration and incubation time (i.e. 50 mM and 300 min). The higher resilience of *P. oleovorans* could be explained by their higher metabolic adaptability to stress situations (Moore et al., 2006; Arana et al., 2010). *Pseudomonas* species related to *P. oleovorans* are also described to be more withstanding to perturbations of their ATP metabolism (Ebert et al., 2011). However, a (partially) alternative mode of action for MEA, MMEA and DMEA on *P. oleovorans* should also be considered. MEA for example is described as an inhibitor of choline dehydrogenase (Haubrich and Gerber, 1981), inhibiting the oxidation of choline to betaine aldehyde, which in turn is oxidated to betaine. This could result in a decreased betaine pool in these bacteria, making them more



sensitive to stress conditions (Kempf and Bremer, 1998). The intracellular pools of both choline and betaine are strictly regulated in *P. aeruginosa* and depletion of either pool results in decreased growth under osmotic stress conditions, emphasizing the importance of betaine for bacteria in stress conditions (Fitzsimmons et al., 2012). Wargo et al. (2013) also postulate that betaine accumulation is likely to be important for survival of many Gram negative opportunistic bacteria. This all suggests that inhibition of betaine synthesis can result in decreased viability, not necessarily linked to an immediate ATP depletion. Moreover, this could also explain the effects of MEA at a more neutral pH seen for *P. oleovorans*, although this does than not explain why no effect was seen in *E. coli* at pH 7.5. Further research is clearly needed to elucidate the possible underlying working mechanism(s) of alkanolamines against different bacterial species.

Overall, our data on the exposure of *E. coli* to MEA, MMEA or DMEA ([Chapter 3](#)) confirm a clear loss of *E. coli* viability due to exposure to the latter 3 compounds at alkaline pH. The effect is confirmed by an immediate ATP depletion, followed by membrane permeabilization and loss of culturability. In contrast, although choline appeared to cause a mild increase in membrane permeability, viability was not affected. These findings therefore support our proposed hypothesis for the antibacterial effect of glycine, sarcosine and DMG ([Chapter 2](#)).

8 Main Conclusions

- Glycine and its *N*-methylated derivatives sarcosine and DMG (but not betaine) cause *E. coli* viability loss under alkaline stress conditions
- The underlying working mechanism is hypothesized to be related to the intracellular proton scavenging effect of this compounds
- This hypothesis was confirmed by the loss of *E. coli* viability due to exposure to MEA and its *N*-methylated derivatives MMEA and DMEA (but not choline), a related series of compounds, under alkaline stress conditions
- Knowledge on the mode of action of these series of compounds will help to identify the antibacterial potential of other interesting amine-based compounds
- Validation of the concept of viability loss for *E. coli* as a model pathogen provides opportunities to verify this mode of action in other microorganisms



9 Future perspectives

To allow the identification of the underlying working mechanism, all experiments were performed under conditions of nutrient deprivation. For application purposes it is now mandatory to evaluate the effects in more complex media. However, the described working mechanism for *E. coli* provides an innovative concept that can be used for both the recognition of novel candidate antimicrobial compounds and the validation of such effects in other bacterial species.

We already established an antibacterial effect of alkanolamines on important bacterial contaminants of MWF, at least partially based on intracellular proton scavenging, which is relevant as the working pH of these fluids is alkaline (about pH 9.5). This effect should further be validated in a MWF-based matrix. Our hypothesized mechanism also allows the development of novel potential MWF biocides and biostatics, based on our intracellular proton scavenging hypothesis. The advantages of non culture-based viability analysis should also be further exploited in MWF quality control systems.

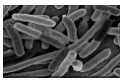
The concept of proton scavenging by amine-based compounds at alkaline pH can however also be of interest to other fields working with alkaline pH conditions. Dental hygiene for example, where alkaline pH is already used as a strategy to treat bacterial biofilms causing tooth decay. Alkaline detergents widely used as cleaning agents, could in our opinion also certainly benefit from the antibacterial effects of amine-based biodegradable antimicrobial compounds.

Extended viability analysis allows the discrimination of different states of injured bacteria and provides substantial evidence to identify the site and even the mode of action of antimicrobial compounds. This can present a substantial benefit to a wide array of industrial applications such as testing the efficacy of sanitizers, monitoring the production of functional foods (probiotics) and assessing water quality.



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Summary



Viability assessment of microorganisms is relevant for a wide variety of applications ranging from fundamental microbiology, over clinical (both human and veterinary medicine), to high throughput industrial applications. It also plays an increasingly important role in the search for novel antimicrobial compounds. As antimicrobial resistance has become a major problem in these fields. Given the adaptability of microorganisms and their tendency to acquire resistance, the development of innovative antimicrobial strategies and the identification of novel antimicrobial compounds has become a continuous challenge.

The **General Introduction** provides an overview both the definitions of bacterial viability and the complementary techniques to assess this viability. It remains one of the major analytical challenges in contemporary microbiology to properly define and identify live, dead and possible intermediate states of bacteria. Moreover, the ability to distinguish these different physiological states is especially important for assessing the survival of pathogenic bacteria after exposure to antimicrobial agents. Reproductive growth is the most stringent proof of viability as it requires both metabolic activity and membrane integrity. However, only the combined data from several complementary methods, including the detection of culturability, provide an adequate level of certainty about the physiological state of a bacterium.

In this PhD thesis we aimed to quantify the effect of glycine and its *N*-methylated derivatives *N*-methylglycine (sarcosine), *N,N*-dimethylglycine (DMG) and *N,N,N*-trimethylglycine (betaine) on the viability of *Escherichia coli* under alkaline stress and to explore the working mechanism of this antibacterial effect based on in-depth viability analysis, targeting different cellular compartments.

In **Chapter 1** we first described the emulsifying potential of glycine and its *N*-methylated derivatives sarcosine, DMG and betaine under varying pH conditions. Oil in water emulsions containing each test compound showed that only DMG is a mild enhancer of emulsification. Subsequently, the effect of all test compounds on the membrane integrity of *E. coli* was evaluated with flow cytometry using the LIVE/DEAD BacLight kit. Results showed a membrane deteriorating effect caused by glycine, sarcosine and DMG, but not by betaine. This effect was pH- and time-dependent and had an apparent threshold at pH 9.0. Alkaline pH itself did not affect membrane integrity. Conventional plate counts confirmed concomitant changes in culturability of the membrane-comprised bacteria: the number of culturable bacteria decreased when incubated with glycine, sarcosine and DMG (but not betaine, nor the saline control) at high pH, confirming the decrease in the percentage of live bacteria shown by flow cytometry. This raised the questions whether the alkaline pH-induced alteration in ionization state of glycine, sarcosine and DMG molecules enables these anions to cause direct membrane damage, or whether the alkaline pH first affects bacterial homeostasis mechanisms, allowing the latter



compounds to affect *E. coli* viability indirectly through structural damage, or whether a combination of both effects takes place?

Chapter 2 focuses on the working mechanism of the antibacterial effect of glycine and its *N*-methylated derivatives on *E. coli* under alkaline stress conditions. Results comparing log phase with stationary phase ([Chapter 1](#)) bacteria first confirmed the loss of culturability due to exposure to glycine, sarcosine and DMG at pH 9.5, assessed by both plate counts and MIC determination. Further analysis of a set of complementary viability parameters revealed an immediate and persistent depletion of cellular ATP during the incubation with glycine, sarcosine, and DMG (but not betaine), at alkaline pH. This was followed by a hyperpolarization of the bacterial cytoplasmic membrane and finally led to a loss of membrane integrity. Betaine did not induce an antibacterial effect on *E. coli* under alkaline stress conditions.

Intracellular pH homeostasis in an alkaline environment places a high energy demand on the cell. Besides an important influence on bacterial homeostasis, the alkaline conditions also have an important influence on the ionization state of glycine, sarcosine and DMG. The ionization state of the trimethylated analogue betaine remains unaffected. In contrast, the amine groups of glycine, sarcosine and DMG are protonated at the near neutral cytoplasmic pH, causing an alkalinization of the cytoplasm by proton consumption. The bacterial cell is now required to spend more ATP to support pH homeostasis. This implies that glycine, sarcosine and DMG, but not betaine, could have the potential to amplify pH stress by scavenging protons within the cytoplasm. A depletion of the ATP pool has many detrimental consequences, as energy is required for numerous cellular reactions, and will also lead to loss of membrane integrity and of culturability.

In **Chapter 3** we aimed to confirm the concept of an antibacterial effect through intracellular proton scavenging by evaluating the antibacterial properties of a parallel series of test compounds. Like glycine, sarcosine and DMG from the series of test compounds, these ethanolamines, i.e. monoethanolamine (MEA), monomethylethanolamine (MMEA) and dimethylethanolamine (DMEA) also have an amine group capable of accepting a proton. The trimethylated derivative choline, which like betaine cannot bind an extra proton on its amine group, was also included. Results confirm our hypothesis for the working mechanism of proton scavengers on *E. coli* under alkaline pH stress. As for the first series, the exposed bacteria showed an immediate ATP depletion, followed by membrane permeabilization and loss of culturability when exposed to MEA, MMEA and DMEA under alkaline conditions, while choline had no effect on ATP concentration and culturability. To further explore the extrapolatability of this concept of intracellular proton scavenging as an antibacterial mechanism to other Gram negative bacteria, *Pseudomonas oleovorans* subsp. *lubricantis* was also incubated with MEA and its *N*-methylated derivatives under alkaline conditions. Results again showed a clear



antibacterial effect, but surprisingly no significant decrease in ATP concentration at pH 9.5 and a concentration of 50 mM test compound. This implies that either *P. oleovorans* is more resilient to perturbations of its cellular ATP metabolism, or that the underlying mechanism does differ from the one postulated for *E. coli*. This difference warrants further investigation.

In the **General Discussion**, the overall major findings of the experimental studies are discussed in a broader context allowing to define general conclusions and future perspectives.

Based on the data gathered assessing a complementary set of viability parameters and based on in-depth literature research, an innovative proton scavenging mechanism is proposed to explain the antibacterial effect of glycine and its mono- and dimethylated *N*-derivatives, sarcosine and DMG, on *E. coli* under alkaline stress conditions. The description of this mechanism offers novel insights that can help identifying potential antibacterial molecules. Validation of this concept for *E. coli* provides opportunities to examine and utilize this mode of action in other microorganisms with relevance in specific applications.

Samenvatting



Betrouwbare viabiliteitsbepaling van micro-organismen is belangrijk voor zowel fundamenteel microbiologisch onderzoek, als geneeskundige (humaan en veterinaire) toepassingen en diverse industriële applicaties. Deze bepaling speelt ook een belangrijke rol in de zoektocht naar innovatieve antimicrobiële componenten. Antimicrobiële resistentie is een belangrijk probleem in zowel de medische (humaan en diergeneeskundig), als de industriële sector. Microorganismen hebben een sterk adaptatievermogen en zijn in staat om resistentie-kenmerken te verwerven. Het ontwikkelen van innovatieve antimicrobiële strategieën en moleculen is dan ook een constante uitdaging geworden.

In de **Algemene Inleiding** wordt een overzicht gegeven van zowel de complexe definitie van bacteriële viabiliteit als van de technieken die kunnen gebruikt worden voor een grondige viabiliteitsanalyse. De identificatie van levende, dode en intermediaire bacteriële populaties is nog steeds een grote uitdaging bij microbiële analyses. De mogelijkheid om verschillende fysiologische toestanden van elkaar te onderscheiden is van groot belang bij het testen van de efficaciteit van antimicrobiële componenten tegenover pathogene bacteriën. Bacteriële groei wordt vaak aanzien als het ultieme bewijs van viabiliteit, omdat het zowel metabole activiteit als de aanwezigheid van een intacte membraan vereist. Evenwel, ook bacteriën die niet meer in staat zijn om te groeien, kunnen nog viabel en zelfs pathogeen zijn. Alleen een combinatie van een aantal complementaire methoden, inclusief groeicapaciteit, biedt de nodige informatie om de fysiologische toestand van een bacterie correct te beoordelen.

De algemene **doelstelling** van dit doctoraatsonderzoek was de beoordeling en kwantificatie van het effect van het aminozuur glycine en zijn *N*-gemethyleerde derivaten *N*-methylglycine (sarcosine), *N,N*-dimethylglycine (DMG) en *N,N,N*-trimethylglycine (betaïne) op de viabiliteit van *Escherichia coli* (*E. coli*) onder alkalische stress condities. Hierbij werd een antibacterieel mechanisme vooropgesteld, gebaseerd op complementaire viabiliteitsbepalingen die de effecten op verschillende cellulaire compartimenten aantoonde.

In **hoofdstuk 1** beschrijven we eerst het emulgerend potentieel van glycine en zijn *N*-gemethyleerde derivaten sarcosine, DMG en betaïne bij verschillende pH waarden. Olie-in-water emulsies gecombineerd met elke component afzonderlijk gaven aan dat alleen DMG kan gezien worden als een potentiële emulsie-verbeteraar. Vervolgens werd het effect van deze componenten op de membraanintegriteit van *E. coli* geëvalueerd aan de hand van flowcytometrie in combinatie met de LIVE/DEAD BacLight kit. De resultaten toonden een membraanbeschadigend effect voor glycine, sarcosine en DMG (maar niet voor betaïne) bij alkalische pH waarden. Dit effect was pH- en tijdsafhankelijk, met een drempelwaarde bij pH 9.0. Alkalische pH op zich had geen effect op de membraanpermeabiliteit. Traditionele bacterietellingen bevestigden een parallel effect op bacteriële



groei. Er was een daling van het aantal cultiveerbare bacteriën wanneer de bacteriële populaties geïncubeerd werden met glycine, sarcosine en DMG bij alkalische pH, in overeenstemming met de daling van het percentage levende bacteriën bepaald met flowcytometrie. Deze resultaten leidden tot de vraag of de pH-geïnduceerde wijziging van de ionisatiegraad van glycine, sarcosine en DMG verantwoordelijk kan zijn voor een direct membraaneffect van deze componenten. Daarnaast is er ook de mogelijkheid dat de pH een negatief effect heeft op de bacteriën, waardoor glycine, sarcosine en DMG in staat zijn de *E. coli* verder te beschadigen. Of is het een combinatie van beide effecten?

In **hoofdstuk 2** wordt de nadruk gelegd op het onderliggende werkingsmechanisme van het antibacteriële effect van glycine en zijn *N*-gemethyleerde derivaten op *E. coli* onder alkalische stress omstandigheden. De resultaten bevestigden een negatieve invloed van glycine, sarcosine en DMG op bacteriële groei en membraanintegriteit. Verdere analyse van een set van complementaire viabiliteitsparameters onthulden een onmiddellijke en persisterende uitputting van de bacteriële energie (ATP) bij blootstelling aan glycine, sarcosine en DMG (maar niet aan betaïne) bij alkalische pH. Dit werd gevolgd door membraanhyperpolarisatie en leidde finaal tot membraanpermeabilisatie. Betaïne had geen antibacteriële invloed op *E. coli* onder alkalische stress omstandigheden.

Homeostase van de intracellulaire pH in een alkalische omgeving vergt veel energie van een bacterie. Naast deze invloed op de bacteriële homeostatische mechanismen, heeft incubatie bij alkalische pH ook een belangrijke invloed op de ionisatiegraad van de aminegroep van glycine, sarcosine en DMG. De ionisatiegraad van het trimethylanalogoog betaïne verandert echter niet. Deze aminegroep van glycine, sarcosine en DMG wordt geprotoneerd bij de overgang van de alkalische extracellulaire omgeving naar het bijna neutrale intracellulaire cytosol. Deze protonconsumptie veroorzaakt een alkalinisatie van het cytosol. De bacterie wordt nu verplicht om nog meer energie te spenderen voor zijn homeostase. Dit houdt in dat glycine, sarcosine en DMG (maar niet betaïne) in staat zijn om pH-stress te verergeren door intracellulaire proton consumptie of zg. “scavenging”. Die cellulaire ATP depletie heeft vele schadelijke gevolgen aangezien energie vereist is voor tal van cellulaire reacties. Ze leidt ook tot een verlies van de membraanintegriteit en de cultiveerbaarheid.

In **hoofdstuk 3** willen we het antibacteriële effect door intracellulaire proton scavenging bevestigen aan de hand van de antibacteriële effecten van een parallelle reeks test componenten. Zoals glycine, sarcosine en DMG uit de eerste reeks, hebben de ethanolamines monoethanolamine (MEA), monomethylethanolamine (MMEA) en dimethylethanolamine (DMEA) ook een aminegroep die kan fungeren als proton acceptor. Het getrimethyleerde derivaat choline kan, in analogie met betaïne, geen proton meer binden op zijn aminegroep. De resultaten bevestigden onze hypothese voor *E. coli*. Om dit concept van een antibacterieel effect door intracellulaire proton scavenging toe te passen in een industrieel relevante applicatie, werd het *E. coli* model uitgebreid naar de voor de



metaalbewerkingindustrie belangrijke kiem, *Pseudomonas oleovorans* (*P. oleovorans*) subsp. *lubrificantis*. Deze werd ook geïncubeerd met MEA en zijn *N*-gemethyleerde derivaten bij alkalische pH. De resultaten toonden opnieuw een duidelijk antibacterieel effect, weliswaar zonder daling van de ATP concentratie. Dit houdt in dat *P. oleovorans* hetzij beter bestand is tegen een verstoring van zijn energie metabolisme, hetzij dat het onderliggende mechanisme deels verschilt van wat we vooropstellen voor *E. coli*. Dit concept dient nog onderzocht te worden.

Gebaseerd op de data uit de verschillende complementaire viabiliteitstechnieken en een grondig uitdiepen van de beschikbare literatuur, konden we een hypothese formuleren over het werkingsmechanisme van het antibacteriële effect van glycine, sarcosine en DMG op *E. coli* onder alkalische omstandigheden. We beschrijven een intracellulair proton scavenging effect van deze componenten dat uiteindelijk leidt tot een verlies van viabiliteit. De beschrijving van dit werkingsmechanisme biedt nieuwe inzichten die kunnen helpen om potentiële antibacteriële moleculen te identificeren. Daarenboven biedt de validatie van dit concept voor *E. coli* uitbreidingsmogelijkheden naar andere pathogene micro-organismen en naar diverse toepassingen.

Curriculum Vitae

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Bibliopgraphy



Curriculum Vitae

Donna Vanhauteghem werd geboren op 21 april 1982 te Oudenaarde.

Na het beëindigen van het hoger secundair onderwijs aan het Koninklijk Atheneum te Oudenaarde, richting Wetenschappen-Wiskunde, startte zij in 2000 met de studies diergeneeskunde aan de Universiteit Gent.

Zij behaalde in 2006 het diploma van Master in de diergeneeskunde (optie gezelschapsdieren) met onderscheiding. Onmiddellijk daarna werkte ze 3 maanden als wetenschappelijk medewerkster aan de faculteit Diergeneeskunde, vakgroep Farmacologie, Toxicologie en Biochemie, onderzoeksgroep Biochemie. Van januari 2007 werkte ze gedurende 2 jaren als laborante bij Envirocontrol N.V. te Wingene. Daar verrichtte ze biochemische analyses op bodem- en grondwaterstalen.

Blijvend geboeid door het wetenschappelijk onderzoek trad ze in februari 2009 in dienst aan de faculteit Diergeneeskunde bij de vakgroep Diervoeding, Genetica en Ethologie. In 2012 behaalde ze een BOF mandaat ter afronding van haar doctoraatsonderzoek bij de vakgroep Farmacologie, Toxicologie en Biochemie. Dit onderzoek betreft de antibacteriële werking van amine gebaseerde moleculen.

Donna Vanhauteghem is auteur van verschillende wetenschappelijke publicaties en abstracts. Ze nam actief deel aan nationale en internationale congressen



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Dankwoord

