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ORIGINAL ARTICLE

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

D. Vanhauteghem · G. P. J. Janssens · A. Lauwaerts · S. Sys · F. Boyen · I. D. Kalmar · E. Meyer

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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) 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 ETEC 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.

Keywords Glycine · Methylamine · Flow cytometry · Membrane integrity · Emulsification · Alkaline stress

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D. Vanhauteghem (✉) · G. P. J. Janssens · I. D. Kalmar
Laboratory of Animal Nutrition, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, 9820 Merelbeke, Belgium
e-mail: Donna.Vanhauteghem@Ugent.be

D. Vanhauteghem · E. Meyer
Laboratory of Biochemistry, Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

A. Lauwaerts
Taminco N.V, Pantserschipstraat 207, 9000 Ghent, Belgium

S. Sys
Department of Internal Medicine and Clinical Biology of Large Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

F. Boyen
Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

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 a 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 *Escherichia 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 ETEC membrane integrity.

Along its path in the digestive tract the ETEC 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 ETEC membrane integrity of glycine and its *N*-methylated analogues.

Materials and methods

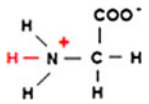
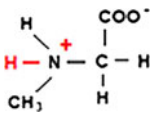
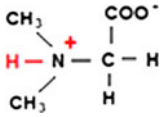
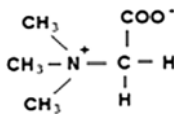
Test compounds

Glycine (≥99% purity), sarcosine (≥99% purity) and betaine (≥99% purity) were obtained from Sigma Aldrich, DMG (≥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.

Emulsifying potential

Emulsifying properties were 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

Table 1 Chemical structures and pKa values of glycine and its *N*-methylated analogues at pH 6.5

	Structure	pKa amine group	pKa carboxylic acid group
Glycine		9.78	2.35
Sarcosine		10.12	2.21
DMG		10.01	1.87
Betaine		–	1.84

DMG N,N-dimethylglycine

500 nm (Genesys 10UV, Spectronic Unicam). The emulsifying stability index (ESI) was calculated using the following equation: ESI (min) = A_0 x ($\Delta T/\Delta A$), where A_0 and A_5 are the absorbances of the diluted oil in water emulsions at 0 and 5 min after homogenization, respectively, $\Delta T = 5$ min and $\Delta A = A_0 - A_5$.

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, 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×*g*, 2 min, 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.

Minimal 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 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 ETEC 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 20 h at 37°C. After incubation, bacterial cells were collected by centrifugation (10,000×*g*, 2 min, 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 SYTO 9 is cell-permeable and can freely enter all ETEC, either live or dead. In contrast, red fluorescent propidium iodide (PI) can only enter membrane-comprised cells. 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 SYTO 9 (5 µM final concentration) and PI (30 µM final concentration) and incubated for 15 min in the dark at RT. Flow cytometric measurements were performed immediately thereafter, using a FACSCanto flow cytometer (Becton, Dickinson and Company, Erembodegem, Belgium). All data on the percentages of live (L), intermediate (I) and dead (D) bacteria (all together approaching 100%) were acquired and processed using FacsDiva 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 20 h incubation with the control condition at each pH, one-way ANOVA was performed and followed by Dunnett multiple comparisons to the control condition. However, when the variances were not homogenous (Levene's test), Welch' robust variation of ANOVA was used 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

Figure 1 presents the emulsifying stability index (ESI) of sarcosine, DMG and betaine, compared to the non-methylated amino acid glycine under varying pH conditions. All

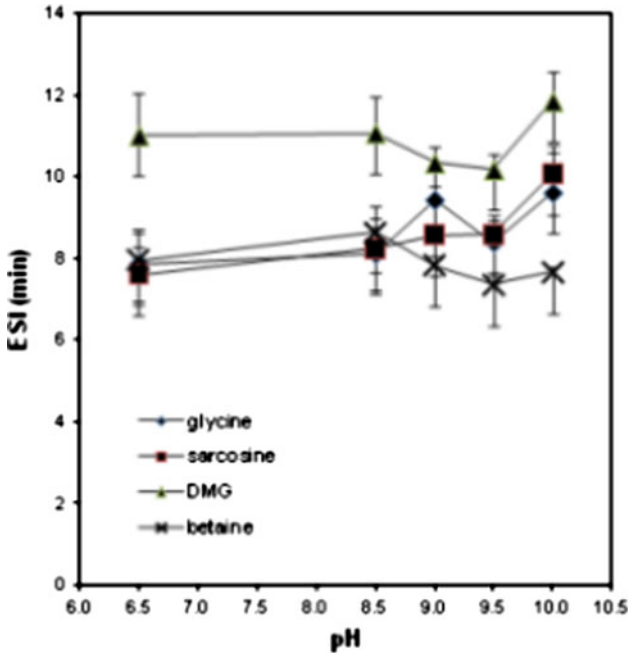


Fig. 1 Emulsifying stability index (ESI) of glycine, sarcosine, DMG and betaine. Data are expressed as means ± SD of triplicate experiments. DMG had the strongest ESI ($p < 0.0005$) compared to glycine, sarcosine and betaine at all pH values tested. There is a significant (positive) regression of ESI 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 ($p = 0.26$)

data are the result of triplicate experiments, mean values ± SD are presented in the online supplementary data table 1.

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 ($p = 0.26$).

Minimal inhibitory concentration (MIC) determination

Concentrations ranging from 1 mM up to 100 mM of each test compound were investigated for antibacterial potential against ETEC. 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 SYTO 9/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. 2, three bacterial subpopulations could be identified: live (L), intermediate (I) and dead (D). With increasing membrane permeability the bacterial population shifts from high a SYTO 9/low PI fluorescence intensity to a state with an even further increased SYTO 9 fluorescence intensity, and then to a state of high PI/low SYTO 9 fluorescence intensity.

Percentages of live, intermediate and dead bacteria are the result of triplicate experiments and all data are presented in the online supplementary data table 2. 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 ETEC samples incubated with DMG for 6 h at the various pH values are presented in Fig. 3, while Fig. 4 presents the live and dead bacterial subpopulations after 20 h of

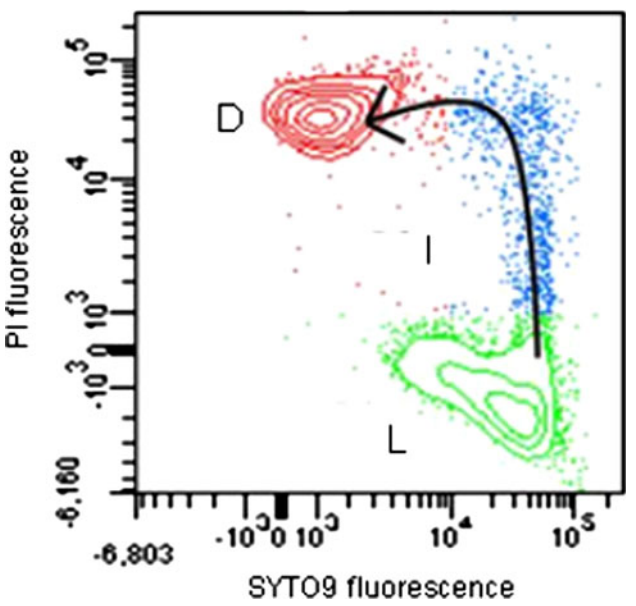


Fig. 2 Flow cytometric SYTO 9/PI dot plot presenting a population of ETEC at different stages of membrane damage. The *arrow* specifies changes in the position of various bacterial subpopulations moving from live (L) over intermediate (I), to dead (D)

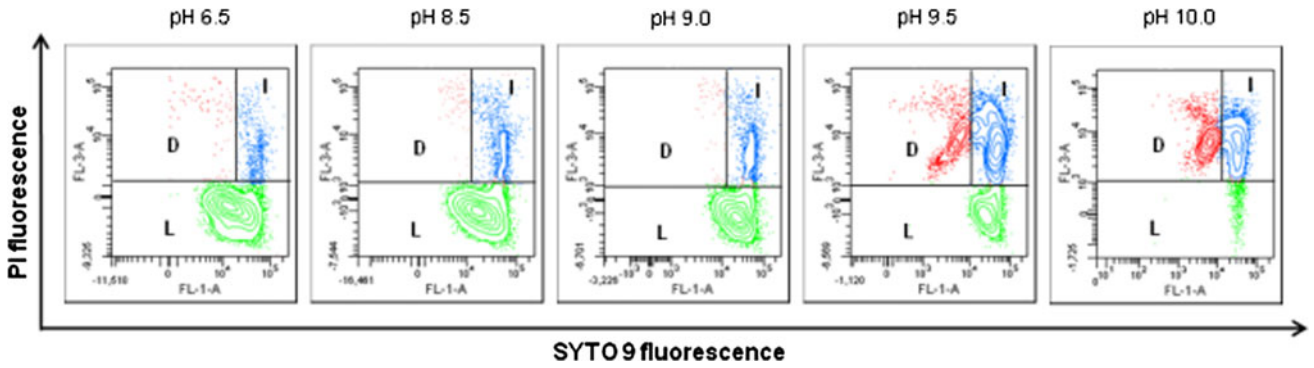


Fig. 3 Flow cytometric SYTO 9(FL1)/PI(FL3) dot plots presenting pH dependent ETEC membrane damage after incubation with 50 mM DMG for 6 h at a pH ranging from 6.5 to 10.0. The L region corresponds to live cells with intact membranes. The I region corresponds to bacteria in an intermediate injured state with comprised membranes. The D region corresponds to dead cells

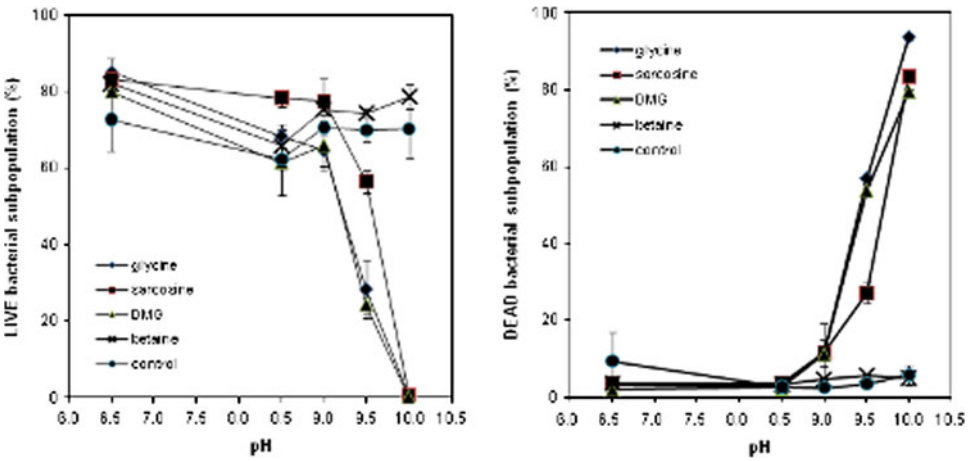


Fig. 4 Percentage of live (L) and dead (D) ETEC subpopulations after 20 h of incubation with glycine, sarcosine, DMG, betaine and sterile saline (control) at a pH ranging from 6.5 to 10.0. Data are expressed as means ± SD of triplicate experiments. There is a significant increase in percentage of dead bacteria in the presence of glycine, sarcosine and DMG at pH 9.0 ($p < 0.05$) and higher ($p < 0.01$)

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.

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 3–6 h when incubated at pH 9.5 and after only 1–3 h when incubated at pH 10.0 (time course data not shown). Table 2 presents the live, intermediate and dead bacterial subpopulations after a 20 h 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 where incubated for 20 h 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.

Table 2 Percentage (mean ± SD) of live (L), intermediate (I) and dead (D) bacterial populations after 20 h of incubation with glycine, sarcosine, DMG, betaine and sterile saline (control) at pH 10.0

	Live	Intermediate	Dead
Glycine	0.8 ± 0.95**	5.9 ± 0.40**	93.7 ± 1.01**
Sarcosine	0.8 ± 0.26**	16.1 ± 1.04**	83.5 ± 1.48**
DMG	0.2 ± 0.12**	21.7 ± 0.81	79.5 ± 0,49**
Betaine	78.8 ± 3.20	16.6 ± 1.65*	4.8 ± 1.86
Control	70.3 ± 7.68	24.2 ± 5.12	5.8 ± 2.56

* $p < 0.05$ (versus control)

** $p < 0.01$ (versus control)

Plate counts

All data are the result of triplicate experiments, mean values ± SD are presented in the online supplementary data table 3. At pH 6.5 and 8.5 there was no significant difference between the plate counts of the ETEC samples incubated with saline, glycine, sarcosine, DMG or betaine.

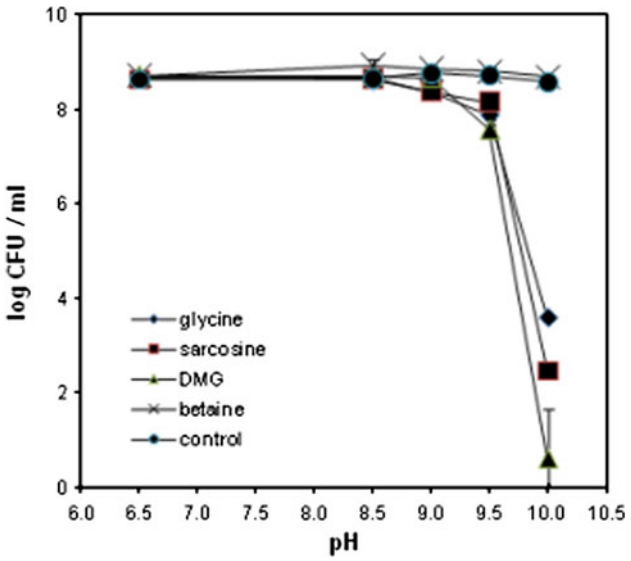


Fig. 5 Culturability of ETEC after exposure to 50 mM of glycine, sarcosine, DMG, betaine and sterile saline (control) for 20 h at a pH ranging from 6.5 to 10.0. There is a significant decrease in CFU/ml when exposed to glycine, sarcosine and DMG at pH 10.0 ($p < 0.01$)

When incubated in more alkaline conditions, there was a pH- and time-dependent decrease in the number of culturable ETEC compared to saline when exposed to glycine, sarcosine and DMG, but not to betaine. The CFU counts of ETEC populations incubated with the different test compounds for 20 h at pH 6.5–10.0 are presented in Fig. 5. Interestingly, differences in colony size in ETEC 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 ETEC sample and no irregularly sized colonies were present at none of the conditions tested.

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 a 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 pKa 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 (Cools et al. 2010; Kalmar et al. 2010a, b; Guan and Tung 1998). Kalmar et al. (2010a) 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 and Onyuksel 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 ETEC 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 ETEC membrane, we used 2-color flow cytometry, based on dual staining with SYTO 9 and PI. This allowed to assess membrane integrity as a main and complementary parameter of bacterial viability (Berney et al. 2006, 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 ETEC membrane deterioration is 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 ETEC 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 20 h at pH 10.0 with each of these three test compounds the portion of live bacteria is <1%, while there are still culturable ETEC counted after plating. This observation suggests that the subpopulation of intermediate bacteria is heterogeneous consisting of culturable and non-culturable ETEC, with varying degrees of membrane damage. The existence of heterogeneity within the intermediate ETEC 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 20 h of incubation also 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 ETEC 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 (Kitko et al. 2010; Wang et al. 2007). 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 ETEC membrane pumps that are required to maintain homeostasis under alkaline conditions. Moreover, our results clearly show that alkaline stress alone does not cause ETEC membrane damage, nor 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 6 h, there is a minor increase in the percentage of intermediates and a decrease in number of culturable bacteria after 20 h of incubation in the control samples. Nevertheless even in these 20 h 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 ETEC more susceptible to the effects of the test compounds when incubated for up to 20 h. 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 ETEC viability due to membrane damage caused by glycine, sarcosine and DMG under alkaline stress conditions, while only DMG could by 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 ETEC 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 ETEC 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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Conflict of interest The authors declare that they have no conflict of interest.

References

Abouseoud M, Yataghene A, Amrane A, Maachi R (2010) Effect of pH and salinity on the emulsifying capacity and naphthalene solubility of a biosurfactant produced by *Pseudomonas fluorescens*. J Hazard Mater 180:131–136

Berney M, Weilenmann H-U, Egli T (2006) Flow cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). Microbiology 152:1719–1729

Berney M, Hammes F, Bosschard F, Weilenmann H, Egli T (2007) Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Appl Environ Microb 73:3283–3290

Clapés P, Infante MR (2002) Amino acid-based surfactants: enzymatic analysis, properties and potential applications. Biocatal Biotransfor 20:215–233

CLSI (2008) Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard, 3rd ed., M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA

Cools A, Maes D, Buyse J, Kalmar ID, Vandermeiren J-A, Janssens GPJ (2010) Effect of N, N-dimethylglycine supplementation in parturition feed for sows on metabolism, nutrient digestibility and reproductive performance. Animal 4:2004–2011

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. Micriobiol Rev 53:121–147

Culham DE, Lu A, Jishage M, Krogfelt KA, Ishihama A, Wood JM (2001) The osmotic stress response and virulence in pyelonephritis isolates of *Escherichia coli*: contributions of RpoS, ProU and other systems. Microbiology 147:1657–1670

Eklund M, Bauer E, Wamatu J, Mosenthin R (2005) Potential nutritional and physiological functions of betaine in livestock. Nutr Res Rev 18:31–48

Eklund M, Mosenthin R, Piepho HP (2006a) Effects of betaine and condensed molasses solubles on ileal and total tract nutrient digestibilities in piglets. Acta Agr Scand A-An 56:83–90

Eklund M, Mosenthin R, Tafaj M, Wamatu J (2006b) Effects of betaine and condensed molasses solubles on nitrogen balance and nutrient digestibility in piglets fed diets deficient in methionine and low in compatible osmolytes. Arch Anim Nutr 60:289–300

Fairbrother JM, Nadeau E, Gyles CL (2005) *Escherichia coli* in post weaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim Health Res Rev 6:17–39

Fan MZ, Adeola O, Asem EK (1999) Characterization of brush border membrane-bound alkaline phosphatase activity in different segments of the porcine small intestine. J Nutr Biochem 10:299–305

Guan J-Q, Tung C-H (1998) Dynamics of fluorescence quenching in pyrene in novel micelles of the zwitterionic betaine surfactant N-(3-dodecyloxy-2-hydroxypropyl)-N, N-dimethylglycine. J Colloid Interf Sci 208:90–95

Kalmar ID, Cools A, Buyse J, Roose P, Janssens GPJ (2010a) Dietary N, N-dimethylglycine supplementation improves nutrient digestibility and attenuates pulmonary hypertension syndrome in broilers. J Anim Physiol An N 94:e339–e347

Kalmar ID, Cools A, Verstegen MWA, Huyghebaert G, Buyse J, Roose P, Janssens GPJ (2010b) Dietary supplementation with dimethylglycine affects broiler performance and plasma metabolites depending on dose and dietary fatty acid profile. J Anim Physiol An N 95:146–153

Kalmar ID, Verstegen MWA, Maenner K, Zentek J, Meulemans G, Janssens GPJ (2011) Tolerance and safety evaluation of *N,N*-dimethylglycine (DMG), a naturally occurring organic compound, as a feed additive in broiler diets. Brit J Nutr. doi: 10.1017/S0007114511004752

Kitko RD, Wilks JC, Garduque GM, Slonczewski JL (2010) Osmolytes contribute to pH homeostasis of *Escherichia coli*. PLoS ONE 5(4):e10078. doi:10.1371/journal.pone.0010078

Kraft ML, Moore JS (2001) Surfactant-induced lysis of lipid-modified microgels. J Am Chem Soc 123:12921–12922

Lallès JP (2010) Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. Nutr Rev 68:323–332

Ly A, Henderson J, Lu A, Culham DE, Wood JM (2004) Osmoregulatory systems of *Escherichia coli*: identification of betaine-carnitine-choline transporter family member BetU and distributions of BetU and trkG among pathogenic and nonpathogenic isolates. J Bacteriol 180:296–306

MacMillan SV, Alexander DA, Culham DE, Kunte HJ, Marshall EV, Rochon D, Wood JM (1999) The ion coupling and organic substrate specifities of osmoregulatory transporter ProP in *Escherichia coli*. Biochim Biophys Acta 1420:30–40

Matthews JO, Southern LL, Higbie AD, Persica MA, Bidner TD (2001) Effects of betaine on growth, carcass characteristics, pork quality, and plasma metabolites of finishing pigs. J Anim Sci 79:722–728

Maurer LM, Yohannes E, Bondurant SS, Radmecher M, Slonczewski JL (2005) pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. J Bacteriol 187:304–319

Metzler-Zebeli BU, Ratriyanto A, Jezierny D, Sauer N, Eklund M, Mosenthin R (2009) Effects of betaine, organic acids and inulin as single feed additives or in combination on bacterial populations in the gastrointestinal tract of weaned piglets. Arch Anim Nutr 63:427–441

Mizumori M, Ham M, Guth PH, Engel E, Kaunitz JD, Akiba Y (2009) Intestinal alkaline phosphatase regulates protective surface microclimate pH in rat duodenum. J Physiol 587:3651–3663

Mykytczuk NCS, Trevors JT, Leduc LG, Ferroni GD (2007) Fluorescence polarization in studies of bacterial cytoplasmatic membrane fluidity under environmental stress. Prog Biophys Mol Bio 95:60–82

Nelson ML, Grier MC, Barbaro SE, Ismail MY (2009) Polyfunctional antibiotics affecting bacterial membrane dynamics. Antiinfective Agents Med Chem. 8:3–16

Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: New insights. Biochem Biophys Acta 1717:67–88

Peddie BA, Wood JE, Lever M, Happer DAR, de Zwart F, Chambers ST (2003) Assessment of antimicrobial activity of hydrophilic betaines in osmotically stressed bacteria. Antonie van Leeuwenhoek 83:175–181

Ratriyanto A, Mosenthin R, Jezierny D, Eklund M (2010) Effect of graded levels of dietary betaine on ileal and total tract nutrient digestibilities and intestinal bacterial metabolites in piglets. J Anim Physiol An N 94:788–796

Sánchez L, Mitjans M, Infante MR, García MT, Manresa MA, Vinardell MP (2007) The biological properties of lysine-derived surfactants. Amino Acids 32:133–136

Smirnova G, Oktyabrsky O (1995) Betaine modulates intracellular thiol and potassium levels in *Escherichia coli* in medium with high osmolarity and alkaline pH. Arch Microbiol 163:76–78

Snoeck V, Cox E, Verdonck F, Joensuu JJ, Goddeeris BM (2004) Influence of porcine intestinal pH and gastric digestion on antigenicity of F4 fimbriae for oral immunization. Vet Microbiol 98:45–53

Stancik LM, Stancik DM, Schmidt B, Barnhart DM, Yoncheva YN, Slonczewski JL (2002) pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. J Bacteriol 184:4246–4258

Sträuber H, Müller S (2010) Viability states of bacteria — Specific mechanisms of selected probes. Cytometry 77A:623–634

Tøndervik A, Strøm AR (2007) Membrane topology and mutational analysis of the osmotically activated BetT choline transporter of *Escherichia coli*. Microbiology 153:803–813

Walker RI, Steele D, Aguado T, the ad hoc ETEC Technical Expert Committee (2007) Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic *Escherichia coli* (ETEC) disease. Vaccine 25:2545–2566

Wang Y, Wang L, Sun Y, Chen Y, Zhu L, Guo L, Luo B, Wang H (2007) Disrupted ompC causes osmosis sensitivity of *Escherichia coli* in alkaline medium. J Genet Genomics 34:1131–1138

Wu WU, Hettiarachchy NS, Qi M (1998) Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. J Am Oil Chem Soc 75:845–850

Xia WJ, Onyuksel H (2000) Mechanistic studies on surfactant-induced membrane permeability enhancement. Pharm Res 17:612–618

Yokoyama S, Kouchi J, Tabohashi T, Harusawa F, Yamaguchi A, Sakai H, Abe M (2001) Emulsifying potency of new amino acid-type surfactant (II): stable water-in-oil (W/O) emulsions containing 85 wt% inner water phase. Chem Pharm Bull 49:1331–1335

Zajic JE, Panchal CJ (1976) Bio-emulsifiers. Crit Rev Microbiol 5:39–66