THE EFFECT OF COPPER, ZINC AND IRON ON THE DEATH RATE OF *SALMONELLA* UNDER ACID CONDITIONS.

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Abstract Liquid pig feed (LPF) and skimmed milk (SM) acidified to pH 4 with lactic or hydrochloric acid (HCl) were treated with 0, 2.5, 5. 12.5, 25 or 50 ppm Cu^{2+} and challenged with 10^7 c.f.u ml^{-1} *Salmonella* Typhimurium DT104:30. The decimal reduction time (D) was determined by enumeration of viable Salmonellae at appropriate time intervals after inoculation. Additional SM samples, acidified to pH 2.5 with HCl were treated with 10 ppm Cu^{2+} , 20 ppm Zn^{2+}, 20 ppm Fe^{2+} or combinations of these, and challenged with *Salm.* Typhimurium DT104:30. In LPF and SM at pH 4.0 increasing concentrations of Cu^{2+} resulted in a non-linear decrease in D for *Salm.* Typhimurium DT104:30. *Salm.* Typhimurium DT104:30 died significantly ($P<0.05$) faster in SM at pH 2.5 treated with Cu^{2+} or Cu^{2+} plus Fe^{2+} compared with SM treated with HCl alone. However, Zn^{2+} or Cu^{2+} plus Zn^{2+} had no significant effect on the death rate. Treating acidified SM with Fe^{2+} alone resulted in a 3 fold increase in the survival of *Salm.* Typhimurium DT104:30.

Introduction Copper sulphate has been added to pig feeds as a growth promoter for many years. Its mode of action is unclear. However, it has been suggested that it may have a selective antimicrobial effect on gut microflora { (Fuller *et al.,* 1960; Shurson *et al.*, 1990). Recent work by Beal *et al* (2004) suggested a mode of action for this affect. They found that the presence of 50 ppm copper ions in acidified liquid feed increased the death rate of *Salm.* Typhimurium DT104:30 by up to 10 fold. (50ppm Cu^{2+} in liquid feed 280g kg^{-1} dry matter is equivalent to 175 mg kg^{-1} dry matter). In addition in liquid feed acidified to pH 4 the death rate of *Salm.* Typhimurium DT104:30 increased with increasing concentrations of Cu^{2+}. These studies demonstrated that copper ions increase the death rate of *Salmonella* in substrates acidified with organic acids (lactic and acetic). However, it was not clear whether the effect was due to the acidity i.e. presence of H^+ or whether it was due to copper interacting with the anion or undissociated acids.

The ability of *Salmonella* and other enteropathogens to survive acidic conditions is an important factor in their pathogenicity. Acid tolerance confers benefits to the organism in terms of its ability to survive the acid barrier of the stomach. The main acid barrier is created in the stomach by the secretion of hydrochloric acid. However, in pigs a considerable contribution to the barrier function may be made by lactic acid produced by lactic acid bacteria inhabiting the proximal stomach (Hansen, 2004). Hansen (2004) demonstrated that pigs fed diets that enhance lactic acid levels in the proximal stomach (coarse ground meals) have reduced incidence of *Salmonella* infections.

In Europe the Scientific Committee for Animal Nutrition (SCAN) (2003) have recommended that the inclusion level of copper in pig diets should be reduced to 4 to 10 mg copper per kg dietary dry matter for grower finisher pigs, with the current maximum of 175 mg kg^{-1} being retained for the first 10 weeks of life only. If the action of copper ions in the presence of lactic acid has such a profound affect on the survival of Salmonellae, the level of copper in the diet could have an affect on the ability of Salmonellae to survive passage through the stomach.

Copper is a redox active metal and other divalent redox active metals such as zinc and iron are also added to pig diets. If the effect of copper on the death rate of *salmonella* is due to its redox activity then it may be supposed that other redox active divalent metals may have a similar effect.

The objective of this paper was to examine some of the interactions between divalent metal ions, principally copper, and acids on the death rate of *Salm.* Typhimurium DT104:30.

Materials and Methods *Salm.* Typhimurium DT104:30 was selected for these studies as it is a particularly acid tolerant. All media were obtained from Oxoid Ltd (Basingstoke, Hampshire, UK).

A series of studies were conducted to examine the effect of copper ions (Cu^{2+}) on the death rate of *Salm.* Typhimurium DT104:30 in acidified liquid pig feed (LPF) and skimmed milk (SM). SM was used as a convenient food substrate for these studies.

Study 1: Sterile LPF (25kgrays γ radiation) was acidified with 200mmol lactic acid L^{-1} (pH 4.0±0.5). Sterile SM (steam sterilized) was acidified with lactic or hydrochloric acid to give a pH of

4.0±0.5. To both substrates copper sulphate was added to give Cu^{2+} concentrations ranging from 0-50 ppm.

Study 2: Samples of SM were acidified to pH 2.5 to mimic the pH conditions in the stomach and treated with 10 ppm Cu^{2+} (from copper sulphate), 20 ppm Fe^{2+} (from ferric sulphate) or 20 ppm Zn^{2+} (from zinc sulphate). The latter two metal ions were included to examine the effects of other redox active metals on the survival of *Salm.* Typhimurium DT104:30 under acid conditions.

In all studies the survival of *Salm.* Typhimurium DT104:30 was assessed as follows. *Salm.* Typhimurium DT104:30 (20 hour culture at 37°C in brain heart infusion (BHI) broth) was added to triplicate 100 ml samples of the substrates to give an initial inoculum of *ca.* 10^7 c.f.u./g. After inoculation LPF and SM samples acidified to pH 4 (the recommended temperature for the production of fermented liquid feed) were incubated at 30°C and SM samples acidified to pH 2.5 were incubated at 37°C. At appropriate time intervals after inoculation 10 ml aliquots were removed and serial (10 fold) dilutions performed in buffered peptone water (BPW). Appropriate dilutions were plated onto blood agar (LPF samples) or BHI agar (SM samples) and incubated at 37°C for 24 h after which colonies of viable *Salmonella* were enumerated and survival curves plotted. The death rate of *Salm.* Typhimurium DT104:30 was expressed as the decimal reduction time (D) which was calculated as the reciprocal of the slope of the survival curve.

For study 1 values of D were plotted against Cu^{2+} concentration, the data were analysed by regression analysis and curves fitted to the data using Genstat 7.2 (Lawes Agricultural Trust, Rothamstead UK). For study 2 data were analysed by analysis of variance using Genstat 7.2.

Results In non-acidified LPF with or without the addition of 50ppm Cu^{2+} numbers of *Salm.* Typhimurium DT104:30 increased from 7.02 to 9.09 and from 7.06 to 8.90 c.f.u. ml^{-1} respectively, indicating that copper ions alone had no antimicrobial effect on this organism in LPF. In LPF and SM acidified to pH 4.0±0.5 with lactic acid (LPF and SM) or hydrochloric acid (SM) increasing concentrations of Cu^{2+} resulted in a non-linear decrease in D for *Salm.* Typhimurium DT104:30 (Table 1). The effect was most apparent in acidified SM where the addition of even a low level of Cu^{2+} (2.5 ppm) resulted in a 5-6 fold decrease in D. In the absence of Cu^{2+} *Salm.* Typhimurium DT104:30 died 3.5 times faster in SM acidified with lactic acid compared with HCl at the same pH.

Salm. Typhimurium DT104:30 died significantly faster in SM acidified to pH 2.5 and treated with 10ppm Cu^{2+} or a combination of 10 ppm Cu^{2+} and 20 ppm Fe^{2+} compared with SM treated with HCl alone. On the other hand 20 ppm Zn^{2+} or a combination of 10 ppm Cu^{2+} and 20 ppm Zn^{2+} had no significant effect on the death rate. Treating acidified SM with 20 ppm Fe^{2+} resulted in a *ca.* 3-fold increase in the survival of *Salm.* Typhimurium DT104:30 (Table 2).

Discussion In SM acidified with lactic or hydrochloric acid there was a dose response in D of *Salm.* Typhimurium DT104:30 to increasing copper concentrations. This suggests that the observed effect is due to the presence of H^+ rather than lactate anions or undissociated lactic acid molecules. However, in the absence of Cu^{2+} *Salm.* Typhimurium DT104:30 died faster because lactic acid is more antimicrobial per se than HCl. The effect of added copper varies between different organic matrices because; low inclusion levels of copper (2.5 ppm) had a far greater effect on the D of *Salm.* Typhimurium DT104:30 in SM than in LPF. In LPF and SM acidified to the same pH with lactic acid the addition of 2.5 ppm Cu^{2+} resulted in a decrease in D of 22 min in LPF compared with 86 min in SM. Whether this was

Cu^{2+} ppm	LPF+Lactic acid*	SM+lactic acid	SM+HCl
0	142.2 (± 11.31)	106.6 (± 13.73)	385.6 (± 67.80)
2.5	120.1 (± 9.23)	20.2 (± 0.54)	65.6 (± 4.63)
5	115.2 (± 9.15)	10.7 (± 1.56)	34.4 (± 4.20)
12.5	83.0 (± 1.59)	7.9 (± 0.53)	13.0 (± 2.20)
25	53.4 (± 2.47)	3.6 (± 0.36)	5.5 (± 0.71)
50	25.4 (± 5.22)	2.3 (± 0.78)	4.3 (±0.29)
Fitted curve for D	-32.5+173.1/ (1+0.04[Cu2+])	1.85+104.7/ (1+1.92[Cu2+])	-1.5+387.1/ (1+1.93[Cu2+])
Percent variance accounted for	97.1	96.9	98.2

Table 1 Mean Decimal reduction time (min) and fitted curves for *Salm.* Typhimurium DT104:30 in liquid pig feed (LPF) or skimmed milk (SM) acidified to pH 4.0±0.5 with increasing concentrations of copper ions from copper sulphate. Figures in brackets represent the standard deviation. *data adapted from Beal *et al* (2004)

ORAL PRESENTATIONS

due to differences in the ability of these substrates to bind acid and/or copper or to differences in their protective effect towards bacterial cells is uncertain.

The additional bacteriocidal ability of Cu^{2+} prevails at pH 2.5 which is more typical of the pH found in the stomach. What is interesting about these results is the affect that Fe^{2+} has on the survival of *Salm.* Typhimurium DT104:30. The organism survived for 3 times longer in the presence of 20 ppm Fe^{2+}. However, if Cu^{2+} was also present the organism died as quickly as with Cu^{2+} alone. The effect of Fe^{2+} is not altogether surprising as iron uptake is linked to acid tolerance in Salmonellae (Foster, 1995). Clearly, there is an interaction between Cu^{2+} and Fe^{2+} that effectively neutralises the effect of Fe^{2+} on the survival of *Salmonella*. Interestingly there is also an interaction between Zn^{2+} and Cu^{2+}. The presence of Zn^{2+} had no effect on the death rate of *Salm.* Typhimurium DT104:30 compared with HCl alone. It also appeared to neutralise the effect of Cu^{2+} as the combination of Zn^{2+} and Cu^{2+} had no significant effect of D compared with Zn^{2+} or HCl alone. Copper and zinc often work together in the cell and zinc appears to have a role in maintaining copper homeostasis in Gram negative bacteria (Rensing and Grass, 2003).

The mechanism by which copper and acid interact to have such a large affect on the viability of *Salm.* Typhimurium DT104:30 is not clear. Small quantities of free Cu^{2+} are toxic to many groups of bacteria (Goto *et al.*, 1993). Beal *et al* (2004) reported that acid stress appeared to decrease the tolerance of *Salm.* Typhimurium DT104:30 to Cu^{2+}, as it was not sensitive to 20.5 ppm free Cu^{2+} at pH 6.16 but was extremely sensitive to lower quantities of free Cu^{2+} at lower pH's.

The current European regulations restrict the addition of copper to grow/finish pig diets to 6 mg kg^{-1}(6 ppm) and the studies reported here suggest that even at this low level there is some advantageous effect in enhancing the barrier function of the stomach. However, the consistency and organic matter profile in the stomach of the pig would bear a greater similarity to LPF rather than SM and in this type of matrix low levels of Cu^{2+} are not nearly as effective.

Conclusion The addition of copper ions to pig diets may reduce the transmission of *Salmonella* by enhancing the acid barrier function of the stomach.

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ORAL PRESENTATIONS

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Metal ion (ppm)	Decimal reduction time (min)
None	6.15[a] (± 0.18)
Cu^{2+} (10 ppm)	4.10[b] (± 0.05)
Zn^{2+} (20 ppm)	6.15[a] (± 0.18)
Zn^{2+} (20 ppm) + Cu^{2+} (10 ppm)	6.60[a] (± 0.13)
Fe^{2+} (20 ppm)	17.21 (± 0.46)
Fe^{2+} (20 ppm) + Cu^{2+} (10 ppm)	4.20[b] (± 0.03)

Table 2 Mean Decimal reduction time (min) of *Salm.* Typhimurium DT104:30 in skimmed milk acidified to pH 2.5 with hydrochloric acid in the presence or absence of metal ions [a,b]means are not significantly different (P>0.05)