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5 Title:

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7 Comparing the antimicrobial effectiveness of NaCl
8 and KCl with a view to salt/sodium replacement

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21 Running title: Salt replacement

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24 **Abstract**

25 A study using a small range of pathogenic bacterial species (*Aeromonas hydrophila*, *Enterobacter*
26 *sakazakii*, *Shigella flexneri*, *Yersinia enterocolitica* and 3 strains of *Staphylococcus aureus*) has
27 shown that potassium chloride has an equivalent antimicrobial effect on these organisms when
28 calculated on a molar basis. Combined NaCl and KCl experiments were carried out and data
29 analysed using a modification to the Lambert and Lambert (2003) model for combined inhibitors
30 (J. Appl. Microbiol. 95, 734–743) and showed that in combination KCl is a direct 1:1 molar
31 replacement for the antimicrobial effect of common salt. If this is a general finding then, where salt
32 is used to help preserve a product, partial or complete replacement by KCl is possible.

33

34

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36 **Keywords:** Predictive modelling, hurdles, salt, potassium chloride, NaCl, KCl

37 **1. Introduction**

38 Salt (NaCl) is generally added to foodstuffs to 1. improve taste and 2. as a preserving
39 agent. Indeed, historically, salt was among the very few effective preserving methods
40 known. With the advent of refrigeration, better processing, packaging, transport and
41 storage, there is less need for high salt levels to maintain product integrity. Furthermore,
42 consumers want products with reduced sodium levels (e.g. due to its relationship with
43 hypertension), but where salt has been added as a preservation hurdle, removal or
44 reduction of the salt will reduce shelf-life and could affect safety in more microbiologically
45 fragile products.

46
47 The most obvious replacement for salt (NaCl) in food products is potassium chloride
48 (KCl). [Strong, Foster and Duncan \(1970\)](#) reported that for the growth of *Clostridium*
49 *perfringens*, solute identity had a bearing on the amount of growth for a given a_w , with
50 KCl having a demonstrably greater effect than NaCl. [Beuchat \(1974\)](#), however, reported
51 that at equivalent a_w NaCl and KCl had equivalent effects against *Vibrio*
52 *parahaemolyticus*; it was reported that in fermented meat products, the replacement of
53 NaCl with KCl did not affect the degree of inhibition and or inactivation, but did alter the
54 taste of the foodstuffs ([Gimeno, Astiasaran, and Bello 1999](#); [Gimeno, Astiasaran, and](#)
55 [Bello 2001](#)). More recently, [Boziaris, Skandamis, Anastasiadi, and Nychas \(2007\)](#) have
56 reported that equal-molar concentrations of NaCl or KCl exerted similar inhibitory effects
57 against *Listeria monocytogenes* in terms of lag phase duration, growth or death rate and
58 that NaCl can be replaced by KCl without risking the microbiological safety, with respect
59 to *L. monocytogenes*, of the product. They also stated that in order to confirm this
60 observation as general, a greater number of organisms needs to be studied.

61

62 In the work reported herein, we simply wanted to answer the following question: can KCl
63 be a direct or partial replacement for NaCl? Since the area of investigation is potentially
64 vast, we concentrated our initial efforts on a few species of pathogenic bacteria with which
65 we already had extensive modelling expertise on and which complimented other published
66 work.

67

68 **2. Materials and methods**

69

70 *2.1. Culture Preparation*

71 *Aeromonas hydrophila* (ATCC 7092), *Enterobacter sakazakii* (1387-2NL), *Staphylococcus*
72 *aureus* (ATCC 6538, ATCC 25923 (labeled as ST121 in this report), ST55 (isolated from
73 pasta)), *Yersinia enterocolitica* (ATCC 9610) or *Shigella flexneri* (ATCC 12022) was
74 grown overnight in a flask containing 80 ml tryptone soya broth (TSB; Oxoid CM 129)
75 shaking at 30°C. The cells were harvested, centrifuged to a pellet (512g, 10 mins, 15°C),
76 washed and re-suspended in peptone water (0.1%). A standard inoculum was produced by
77 diluting the culture to an OD of 0.5 at 600nm. This standardized culture was then further
78 diluted in TSB to produce the starting inoculum (approx 1×10^5 CFU ml⁻¹ in the microtitre
79 plate).

80 *2.2. Experimental method*

81 Experiments were carried out either using half-fold dilutions using the method of [Lambert](#)
82 [& Pearson \(2000\)](#) or by using linear dilutions from stock solutions of sodium chloride or
83 potassium chloride for the effect of individual inhibitors or the method of [Lambert and](#)
84 [Lambert \(2003\)](#) for combined inhibitors.

85 *2.3. Data analyses and model fitting*

86 The data obtained from the Bioscreen are tables of optical density (OD) and time. The time
87 to detection was defined as the time to produce an OD =0.2 at 600nm. The assumption
88 being made was that at an OD =0.2, each well had identical numbers of microorganisms.
89 Furthermore, microscopic checks were performed to see if cell elongation occurred at the
90 highest salt levels used: no such elongation was observed. Data were transformed to the

91 reciprocal in order to stabilise data variance. Wells which showed no visible growth during
92 the period of the experiment were removed from the analysis (censored data).

93 Previous publications (e.g. [Lambert and Bidlas 2007](#)) had used a general model for the
94 fitting of time to detection data (TTD). A modified form of this model, which allows for a
95 definitive MIC for individual inhibitors - the linear-exponential model (E-L), was used to
96 analyse the data obtained, Eq.(1).

$$\begin{aligned} & \text{If } [x] = 0, \text{RTD} = P_0 \\ & \text{Else if } [x] < [P_1], \\ & \text{then } \text{RTD} = P_0 \exp\left(-\left(\frac{[x]}{P_1}\right)^{P_2}\right) \\ & \text{Else, } \text{RTD} = \frac{P_0}{e} \left(1 - P_2 (\log_e [x] - \log_e P_1)\right) \end{aligned} \quad (1)$$

103 Where $[x]$ is the concentration of the inhibitor, P_i are experimental parameters and e
104 is the exponential, RTD is the reciprocal of the time to detection (min^{-1}).

106

107 The minimum inhibitory concentration was calculated from the parameter values obtained
108 for each inhibitor using

$$MIC = P_1 \exp\left(\frac{1}{P_2}\right) \quad (2)$$

110 For combined inhibitors (combinations of NaCl and KCl) the model of [Lambert](#)
111 [and Lambert \(2003\)](#) was modified in a similar way to Eq.(1) allowing a definitive growth-
112 no growth boundary to be constructed for combinations, Eq.(3)

113

$$\left\{ \begin{array}{l}
 \text{if } \sum_{i=1}^n [x_i] = 0, RTD = P_0 \\
 \text{else if } \ln[EffC] < 0 \\
 \text{then } RTD = P_0 \exp(-[EffC]^{P_c}) \\
 \text{else } RTD = \frac{P_0}{e} (1 - P_c \ln[EffC])
 \end{array} \right. \quad (3)$$

114 Where $[EffC] = \sum_{i=1}^n \left(\frac{[x_i]}{P_{2i-1}} \right)^{P_{2i}}$ and P_c is the multinomial exponent for the combined system

115 (Lambert and Lambert 2003).

116 Data were fitted to the equation using non-linear regression using the minimised sum of
 117 squares as the search criterion. Analyses were done using the JMP Statistical Software

118 (SAS Institute Cary, NC).

119

120 3. Results

121 3.1. Growth inhibition by sodium chloride and potassium chloride

122 Three species – the Gram negatives *E. sakazakii* and *A. hydrophila* and the Gram positive
123 *S. aureus* are used below to highlight the results obtained. Three strains of the latter
124 organism were used, due to the importance of humectant activity to control the growth of
125 this organism. The results from these and the other two organisms examined in this study
126 are summarised in [Table 1](#).

127

128 3.2 *Enterobacter sakazakii*

129 Optical density/incubation time data were collected using half-folded dilutions of NaCl or
130 KCl. When analysed using the modified RTD model there was an excellent fit; [Figure 1](#)
131 shows the results for the effect of KCl on the RTD. At KCl concentrations less than 1×10^4
132 mg l^{-1} (1 %), there is little effect on the rate to detection, i.e. shows uninhibited growth.
133 Above 1%, inhibition increases and above $1.2 \times 10^5 \text{ mg l}^{-1}$ (12%) KCl no growth was
134 observed.

135

136 [Table 1](#) gives the experimental parameters found in percent and in mol l^{-1} . In terms of mol
137 l^{-1} the MIC of NaCl and KCl are within the 95% confidence interval of the mean
138 (calculated from the parameters P_1 and P_2) given for each humectant. [Figure 2](#) compares
139 the observed and fitted data for both the NaCl and KCl inhibition. From these results, when
140 expressed in mol l^{-1} , there is no evidence that NaCl and KCl have different inhibitory
141 effects against *E. sakazakii*. It was also found that in both cases there was evidence of a
142 threshold concentration of approx. 0.1M added salt before any observation of growth
143 inhibition.

144

145 3.3. *Staphylococcus aureus*

146 Comparison of NaCl and KCl were carried out on three strains of *S. aureus* (two standard
147 strains and a factory isolate). [Figure 3](#) shows the results of both experiments in terms of
148 mol l⁻¹ for strain ST121. The time to detection was transformed using the natural logarithm.
149 The curvature observed in Fig. 3 is typical for salt inhibition. Similar results were found
150 for the other two *S. aureus* strains examined.

151

152 It can also be observed from the figure that as conditions become more harsh, the
153 variability (or observed error) in the time to detection also increases, even although the
154 logarithm transformation has been used. There is a general observation throughout
155 predictive microbiology that the variance increases as conditions become harsher and
156 without variance stabilisation the accuracy of models is reduced the closer they approach
157 the MIC of the preservative. In this particular case the reciprocal transformation performs
158 better than the logarithmic.

159

160 3.4. *Combination NaCl and KCl experiments*

161 If the hypothesis that NaCl and KCl are mutually replaceable is correct, then the
162 antimicrobial effect of combinations of NaCl and KCl should be predictable. If the
163 calculation is performed using a combined humectant concentration in terms of mol⁻¹ then
164 the modelling is facile. If, however, the separate identities are kept, then a complication
165 arises due to the non-unity dose response. Previous work done on combined hurdles has
166 used the following equation to describe the effect on RTD (Lambert and Bidlas 2007)

$$167 \quad RTD = P_0 \exp \left(- \left(\sum_{i=1}^n \left(\frac{[x_i]}{P_{2i-1}} \right)^{P_{2i}} \right) \right) \quad (4)$$

168 For combined NaCl and KCl equation (4) will only give an approximation to the observed
169 pattern of inhibition because the parameter P_{2i} for both humectants is approximately 2 and
170 the above equation ignores the binomial expansion of the combined response. Equation (4),
171 which takes this into account was used to examine the observed RTD data for *A.*
172 *hydrophila* from a chequerboard of NaCl/KCl mixtures (observed data [Figure 4](#), modelled
173 data [Figure 5](#)). There is a very good fit of the model to the observed data. [Figure 6](#) shows a
174 plot of the observed and modelled RTD against the Effective Concentration; that the *EffC*
175 is made up from any combination of NaCl and KCl and that the observations all lie on or
176 close to the modelled line is a simple graphical representation to show that there is no
177 synergy between the two inhibitors, since any ‘true’ synergy would cause a mismatch.
178 Further, the predicted values for the combined experiment (as opposed to the direct
179 modelling of the observed data) are given in [Table 2](#) for both the *A. hydrophila* and the
180 identical *E. sakazakii* experiments. The predicted parameters are in agreement with those
181 modelled from the observed data.

182 4. Discussion

183 This work focuses on the ability of potassium chloride to replace salt as an antimicrobial
184 humectant. From this study KCl is a direct 1:1 molar replacement for the antimicrobial
185 effect of common salt. This study enhances the work done by Boziaris et al. (2007) and
186 delivers the same conclusion for a larger spectrum of pathogens. There is still, however,
187 clarification required for some of the other common pathogens as the paper by Strong et al.
188 (1970) would suggest. There is also one point which needs to be made: there is no
189 differentiation in this hypothesis of the equivalence of KCl and NaCl for the antimicrobial
190 effect to be due to the chloride ion. The use of calcium chloride and other similar salts
191 would immediately differentiate these two possibilities.

192

193 The model and the definition used for the effective concentration are direct applications of
194 the Gamma hypothesis ([Zwietering, Wiltzes, De Wit and Van't Riet 1992](#)) - that individual
195 inhibitors act independently against the growth of microorganisms. Recently, attempts had
196 been made to expand the hypothesis to include the possibility of factor interaction, based
197 on the observation of the shape of the Growth/No growth boundary for combined
198 antimicrobials ([Augustin and Carlier 2000](#); Le Marc, Huchet, Bourgeois, Guyonnet, Mafart
199 and Thuault 2002). The model described here adequately describes the shape of these
200 G/NG boundaries without recourse to altering the Zwietering hypothesis.

201

202 [Figure 7](#) shows the calculated Growth/No Growth boundary for combinations of NaCl and
203 KCl for the organisms studied. From equation (3) when the effective concentration is
204 greater than 1, the linear function takes over the description of the level of inhibition. For a
205 given RTD this describes a contour for a twin-mix of inhibitors, a surface for ternary-
206 mixes and hypersurfaces for mixtures with greater numbers of components. The $RTD = 0$
207 contour, i.e. the absolute growth/no growth boundary is given by

$$RTD = 0 = \frac{P_0}{e} (1 - P_c \ln[EffC])$$

208

∴

$$EffC = Exp(1/P_c)$$

209 For any combination of inhibitors where the calculated effective concentration is equal to
210 $Exp(1/P_c)$, this combination will define a point on the MIC contour (surface, etc). Any
211 combination with $EffC > Exp(1/P_c)$ lies in a NG zone, and any with $EffC < Exp(1/P_c)$ lies
212 in a G zone. For a given organism, conditions beyond the calculated boundary line will
213 result in no growth. If total salt concentrations, for a given product, are within a boundary
214 line, then other preservative factors (e.g. temperature or weak acid preservatives) have to
215 be used to ensure no growth.

216

217 Most antimicrobial hurdles examined previously such as pH and weak acids give a linear
218 relationship between the log of the time to detection and the concentration, hence the dose
219 response parameter $P_2 \cong 1$ (Lambert and Bidlas 2007). Combinations of such hurdles will
220 also give $P_c \cong 1$. The humectants used in this study, NaCl and KCl are unusual in that the
221 dose response of each was approximately 2. Dose responses of other antimicrobials can
222 vary substantially e.g. phenolics have dose responses (or dilution coefficients) >6 ,
223 (Lambert and Johnston 2000; Lambert and Lambert 2003).

224

225 Modelling offers a cost-effective approach to understanding the microbial growth response
226 in foods. Indeed Zwietering (Zwietering et al.1996) has said that using a model to predict
227 the consequences of changing a formulation on microbial growth is a factor of 1000
228 quicker than attempting a large scale storage trial. Of course the formulator needs access to
229 the model in the first place and must also have an idea of its robustness. However, the use
230 of mathematical models can help to reduce the need for storage trials, challenge tests,
231 product reformulations and process modifications, which are labour intensive, time

232 consuming and expensive. The above example of NaCl vs. KCl is an example whereby a
233 relatively rapid method of obtaining relevant information in conjunction with a robust
234 model leads to a simple algorithm for NaCl replacement, allowing a product developer to
235 gain insight perhaps 1000 times faster.

236

237 **References**

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287 microbiology to estimate the number of *Bacillus cereus* in pasteurised milk at the point
288 of consumption. International Journal of Food Microbiology 30, 55-70.

290 Table 1. Modelled parameters values for NaCl and KCl inhibition for Pathogens used in this
 291 study

Organism	Humectant	P ₁ (mg l ⁻¹) (SErr)	P ₁ (mol l ⁻¹) (SErr)	P ₂ (SErr)	MIC (mg l ⁻¹)	MIC (mol l ⁻¹) (95% CI)
<i>E. sakazakii</i>	NaCl	48180 (160)	0.824 (0.003)	1.609 (0.010)	89810	1.537 (1.516-1.561)
	KCl	62970 (390)	0.845 (0.005)	1.587 (0.0182)	118200	1.586 (1.545-1.630)
<i>A. hydrophila</i> (ATCC 7966)	NaCl	32290 (330)	0.552 (0.006)	2.080 (0.050)	52230	0.893 (0.857-0.932)
	KCl	41870 (410)	0.562 (0.006)	1.910 (0.0395)	70670	0.948 (0.911-0.987)
<i>Y. enterocolitica</i> (ATCC 9610)	NaCl	32700 (262)	0.560 (0.0045)	2.034 (0.061)	53460	0.915 (0.873-0.961)
	KCl	37930 (172)	0.509 (0.0023)	1.859 (0.030)	64950	0.871 (0.848-0.895)
<i>Sh. flexneri</i> (ATCC 12022)	NaCl	37040 (170)	0.634 (0.003)	2.189 (0.036)	58490	1.000 (0.977-1.025)
	KCl	43410 (291)	0.582 (0.004)	1.673 (0.031)	78900	1.0589 (1.022-1.096)
<i>S. aureus 121</i>	NaCl	89600 (500)	1.533 (0.009)	2.190 (0.0476)	141500	2.421 (2.349-2.498)
	KCl	114900 (554)	1.542 (0.007)	2.114 (0.0366)	184400	2.474 (2.411-2.539)
<i>S. aureus 6538</i>	NaCl	88100 (710)	1.508 (0.012)	2.176 (0.0603)	139500	2.388 (2.292-2.488)
	KCl	115700 (590)	1.552 (0.008)	1.984 (0.0363)	191500	2.569 (2.496-2.645)
<i>S. aureus 55</i>	NaCl	87650 (470)	1.500 (0.008)	1.875 (0.0341)	149400	2.557 (2.482-2.634)
	KCl	114400 (800)	1.534 (0.010)	1.817 (0.0408)	198400	2.661 (2.558-2.770)

292 Table 2. Predicted and fitted parameters for the combined experiment of KCl and NaCl evaluated
 293 using equation 3.
 294

Organism		Parameter	Predicted value	Fitted value
<i>A. hydrophila</i>	NaCl	P₁	32290	32880
		P₂	1.000	1.018
	KCl	P₃	41870	43220
		P₄	0.918	0.942
	Combined	P_c	2.080	2.010
<i>E. sakazakii</i>	NaCl	P₁	48180	47670
		P₂	1.000	1.100
	KCl	P₃	62970	58880
		P₄	0.988	1.033
	Combined	P_c	1.606	1.526

295 P₁ and P₃ values quoted are in mg l⁻¹

296

297 **Legend to Figures**

298

299 Figure 1. Effect of KCl on the rate to detection of *Enterobacter sakazakii* in TSB at 30°C.

300

301 Figure 2. Comparison of the observed (symbols) and modelled (solid line) NaCl and KCl

302 inhibition of *Enterobacter sakazakii*; □ NaCl; ○ KCl. The models for NaCl and KCl are

303 essentially coincident and only that for NaCl is shown.

304

305 [Figure 3](#). Comparison of the observed ln(time) to detection (mins) for NaCl (x) or KCl (○)

306 inhibition of *Staphylococcus aureus* 121(ten replicates per humectant concentration).

307

308 Figure 4. Observed RTD for mixtures of NaCl and KCl inhibition of *Aeromonas*

309 *hydrophila*

310

311 Figure 5. Modelled RTD for mixtures of NaCl and KCl inhibition of *Aeromonas*

312 *hydrophila*

313

314 Figure 6. A plot of the effective concentration against the observed (symbols) and

315 modelled RTD (solid line) for combinations of NaCl and KCl against *Aeromonas*

316 *hydrophila*.

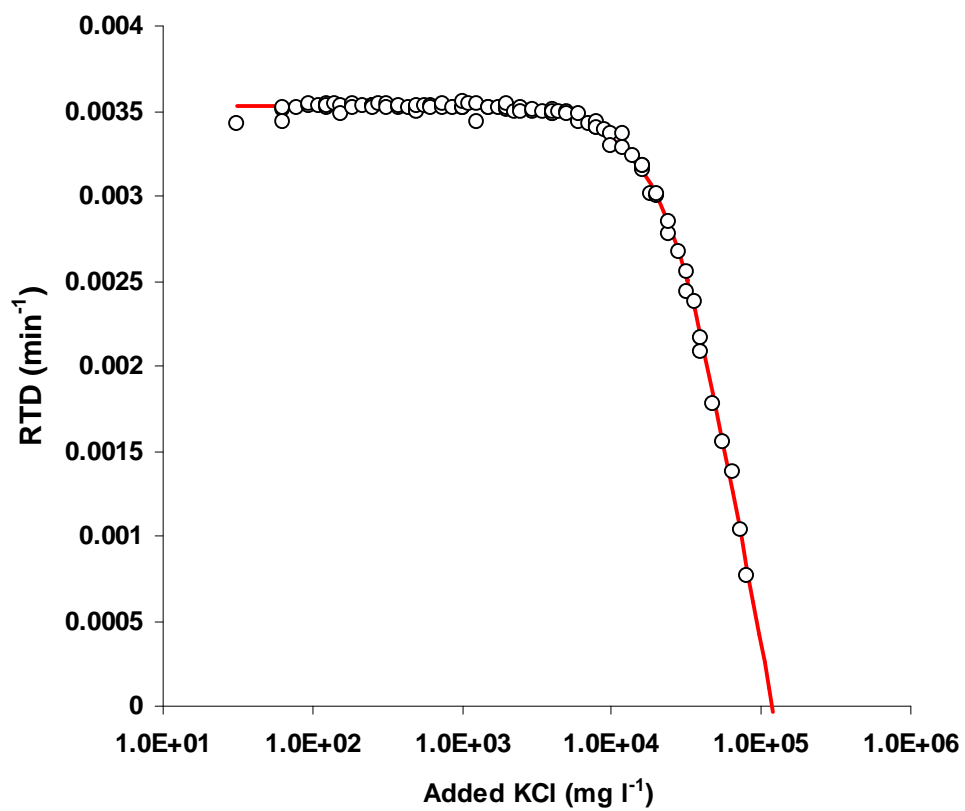
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318 Figure 7. MIC boundaries for NaCl/KCl mixtures; ●, *Staphylococcus aureus* (Saur121);

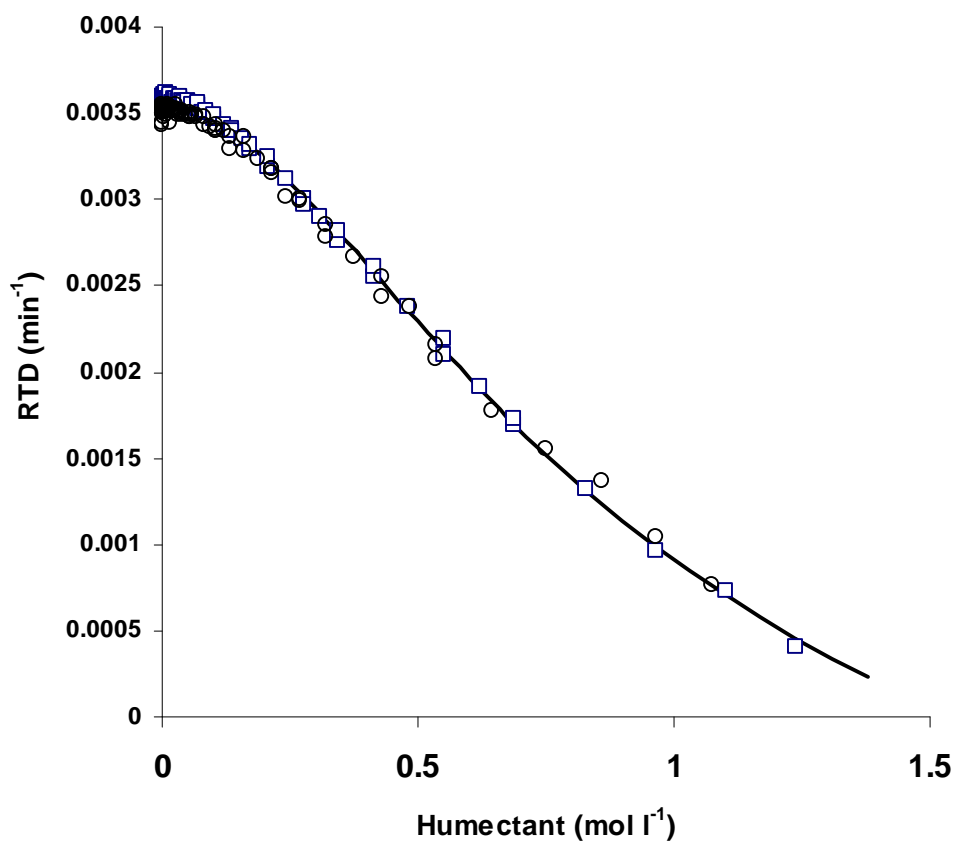
319 ○, *Enterobacter sakazakii*; ■ *Shigella flexneri*; □, *Aeromonas hydrophila*; ▲, *Yersinia*

320 *enterocolitica*.

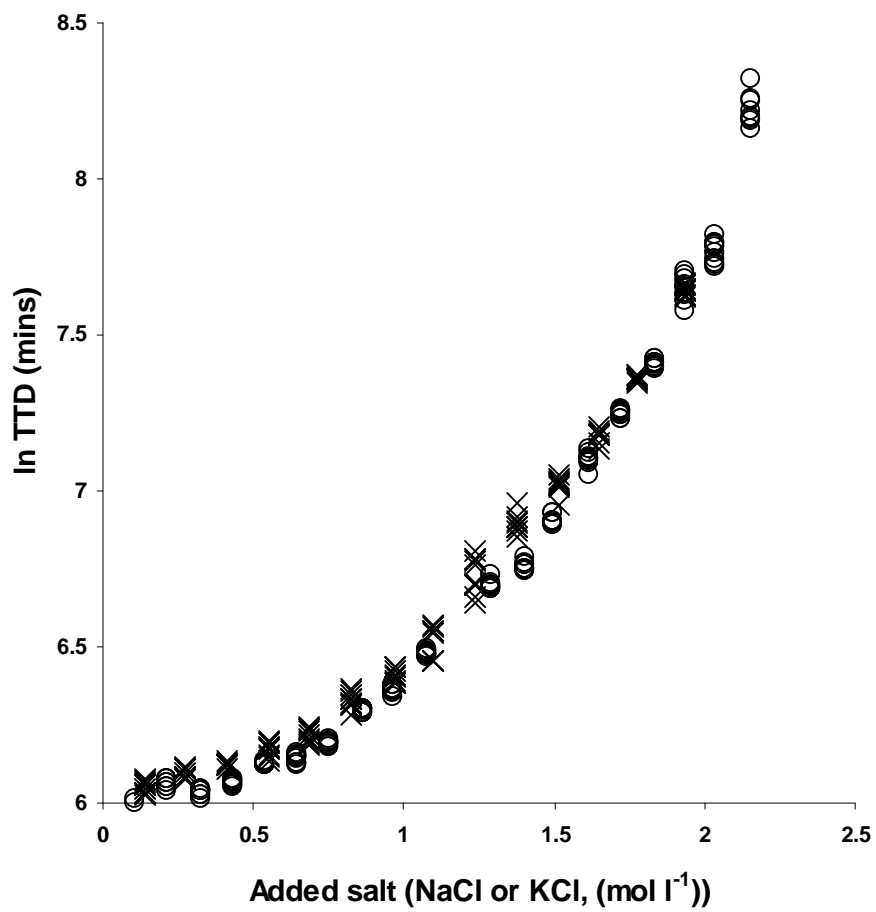
321 *Figure 1.*



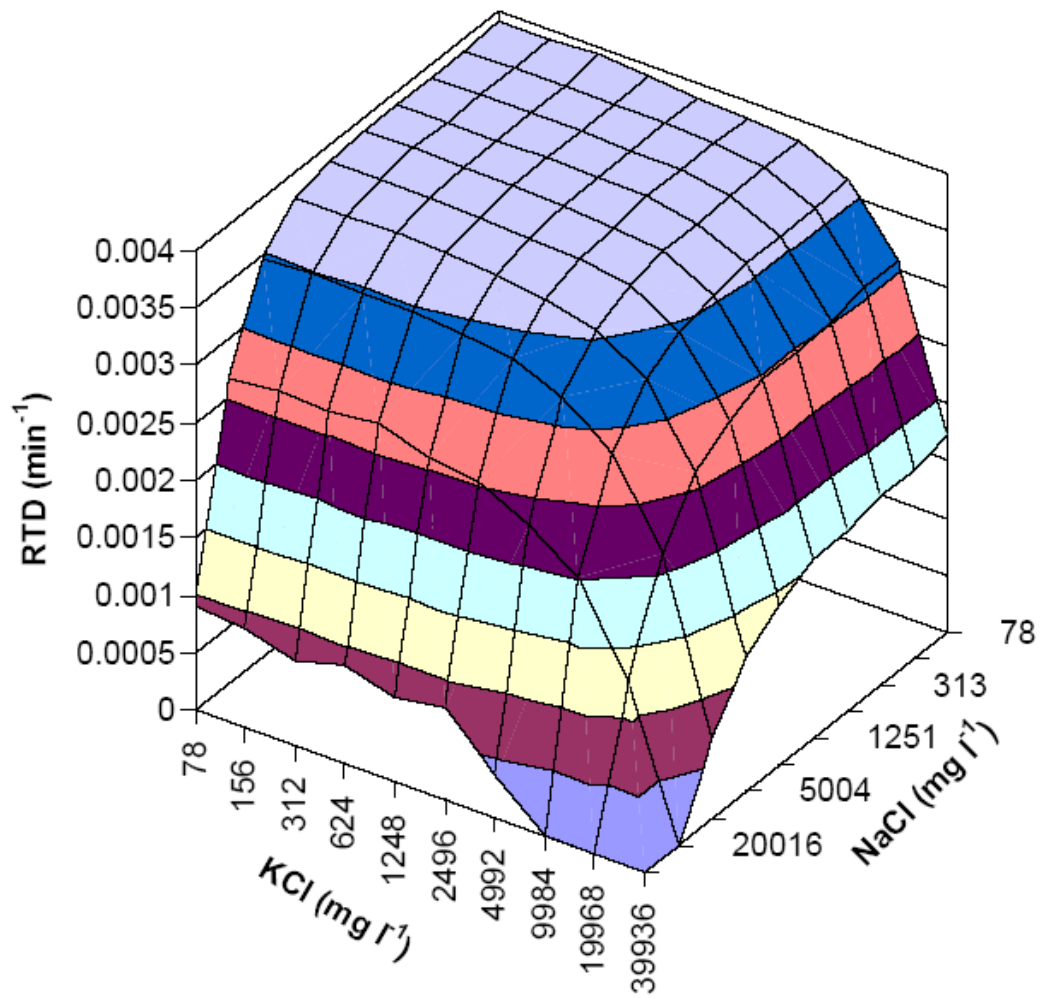
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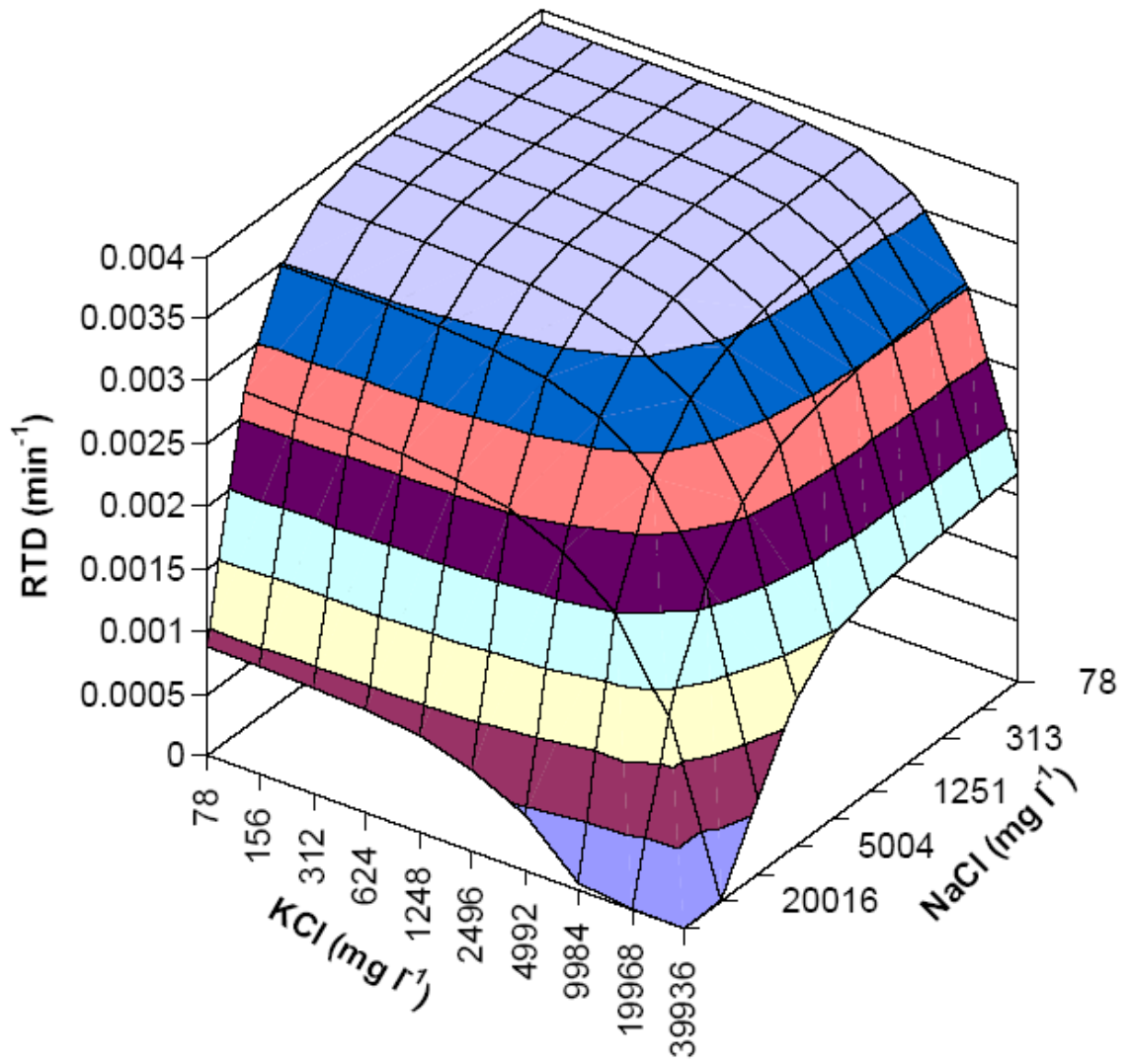


325 *Figure 3.*

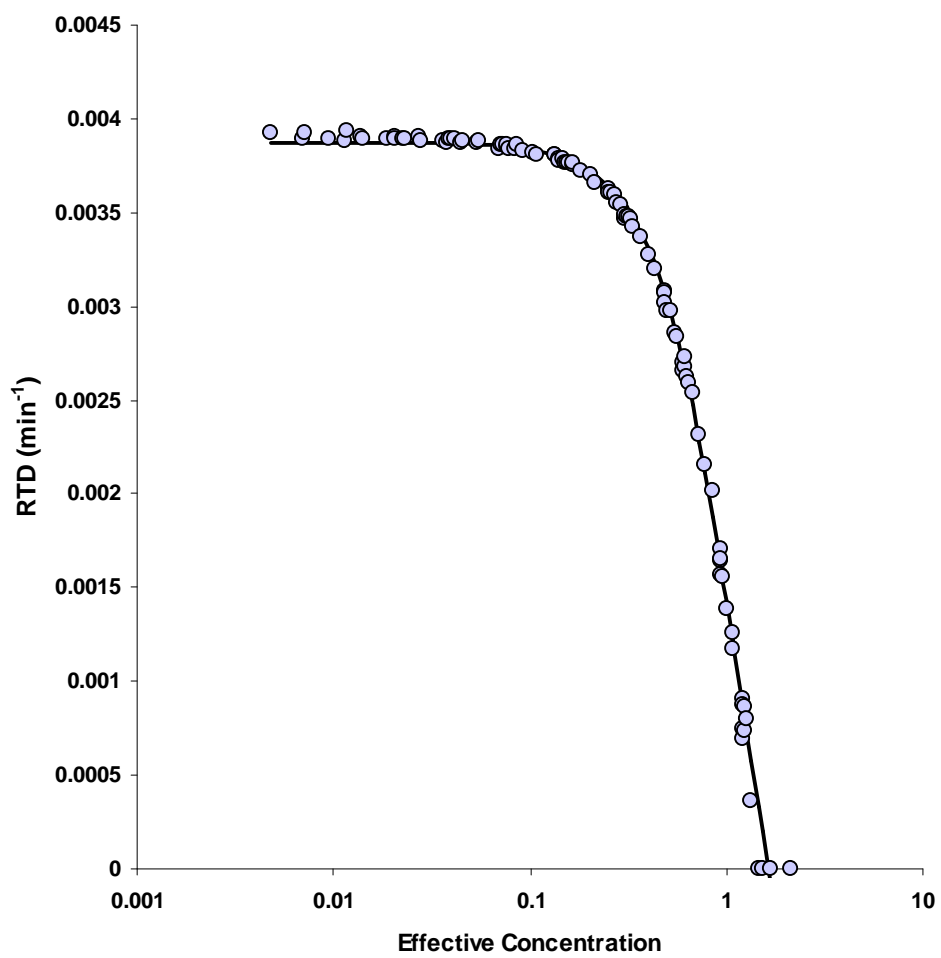


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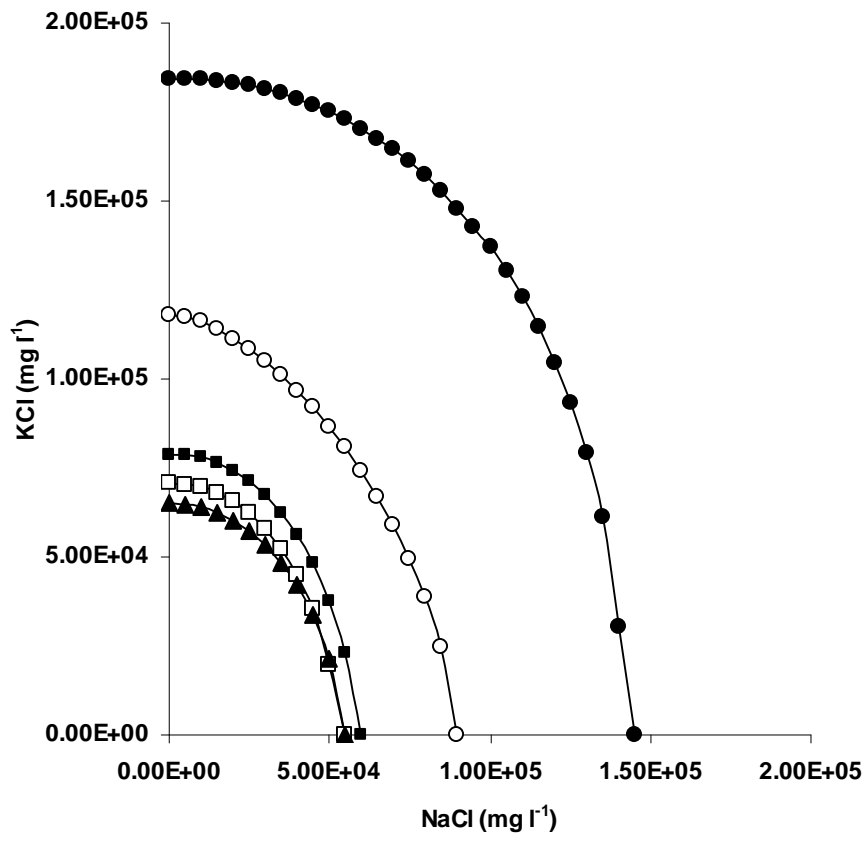


331 *Figure 6.*



332

333 *Figure 7.*



334

Comparing the antimicrobial effectiveness of NaCl and KCl with a view to salt/sodium replacement.

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