RESEARCH REPORT

PORK SAFETY

Title: Effects of pH, temperature, sodium chloride, sodium nitrite and sodium tripolyphosphate on the fate of *Arcobacter* – **NPB #98-157**

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I. Abstract

Arcobacter spp. are considered to be emerging pathogens with potential food safety implications involving raw foods, including pork and poultry products. Prior to this project, little was known concerning environmental factors suitable for growth of the primary pathogen of the genus, *Arcobacter butzleri*, on foods. In this project, the growth characteristics of *A. butzleri,* were studied under various multiple stress conditions in order to develop a means of predicting their growth in environmental and food product situations utilizing these stress conditions. The effect of combinations of temperature, initial pH, sodium chloride (%), sodium nitrite (µg/ml) and sodium tripolyphosphate (%) levels on *A. butzleri* were studied. Growth data generated were fitted to the modified Gompertz function and the Gompertz parameters A, C, M & B were calculated for each treatment combination. Derived growth kinetics: LPD (lag phase duration), GT (generation time), EGR (exponential growth rate) and MPD (maximum population density) values were then calculated for each stress combination.

It was determined that the *A. butzleri* studied are highly sensitive to low levels of sodium tripolyphosphate (STPP) and do not grow or survive at STPP levels above 0.016% and 0.02% respectively. MPDs achieved were largely independent of environmental conditions, and ranged from 8.8-9.2 $\log_{10}$ CFU/ml. When temperature and pH were the two interacting factors, temperature was found to exert a primary influence on LPDs and GTs, with the temperature of 19□C showing the highest values for these variables. Within each temperature, an initial pH of 6.45 was found to have the longest LPD. STPP levels were found to play an important role in extending LPD values, in comparison with sodium nitrite or sodium chloride levels. Some interaction effects were noted between the five variables studied. The data obtained from the curve fitting analysis was used to identify mathematical relationships to predict the growth responses of *A. butzleri* to these environmental stresses.

Studies were then carried out to validate the data obtained from the broth studies into growth responses for *A. butzleri* in raw ground pork. Our studies indicate that the *A. butzleri* strains studied do not grow in raw ground pork under the variable conditions

considered in the validation experiments. Thus, since the organism may survive, but does not increase in numbers under these variable conditions, the primary purpose of the microbiological safety study i.e., to ensure the safety of the food product is fulfilled.

II. Introduction

Organisms belonging to the *Campylobacter*-related genus *Arcobacter* have been clinically associated with cases of gastroenteritis and occasionally septicaemia in humans and with enteritis, abortions and mastitis in livestock (Wesley, 1994). *A. butzleri* is the species most commonly isolated from clinical specimens and foods. *A. cryaerophilus* has also been identified in some environmental isolations, but to a lesser extent as compared to *A. butzleri. Arcobacter* spp. have been recovered from drinking water supplies (both surface & reservoir) and raw food including red meats and poultry (Wesley, 1997).

These organisms have some characteristics in common with *Campylobacter*, including their fastidious growth requirements, as a result of being relatively metabolically inert and lacking significant fermentative capabilities. Foodborne illness associated with *Campylobacter* is considered to be a significant problem in the U.S., the organism has been isolated in 45% of cases of diarrhea (FSIS document). It has been postulated that the role of *Arcobacter* in foodborne illness is not currently well understood, and may be more significant than presently thought, keeping in mind its close relatedness to *Campylobacter* and the similarity of disease syndromes caused. As a result, some cases of foodborne illness thought to be *Campylobacter*-associated, may actually be misidentified cases of *Arcobacter*-related illness.

Predictive microbiology is a useful tool which involves obtaining detailed knowledge about the growth response of microorganisms to environmental conditions, which subsequently allows evaluation of the effects of processing and post-processing conditions on microbial food safety (McMeekin *et al*, 1993). Some studies have determined the growth limits for *Campylobacter* spp., based on which methods can be devised to limit their growth and survival in food products. Published growth limits for *Campylobacter* include a minimum and maximum temperature of 32 and 45□C, respectively; a pH range of 4.9-9, and an NaCl (%) optimum and maximum of 0.5 and 1.5, respectively (ICMSF, 1996). Along the same lines, it would be useful to determine the similarities and differences in growth and survival conditions for *Arcobacter* spp. with a view to predicting their presence in a potentially contaminated food product. This information would be especially useful in food hazard evaluation in a HACCP program.

III. Objectives

The objective of this study was to build a database of information about the growth and survival characteristics of *Arcobacter* spp., in broth media under laboratory conditions, under the influence of combined multiple stress conditions usually encountered by the organisms in the environment and in food products. This knowledge was then extrapolated

using a mathematical model, to predict the growth and survival of the organism in a variety of combined stress conditions, in laboratory culture media and in ground pork.

IV. Procedures

Table 1: Preliminary studies identified the range of growth and survival conditions for *Arcobacter spp.* as follows:

	pH	Temperature	NaCl level (%)
Growth	5.5 – 8.0	10□C-37□C	0.09-3.5%
Survival	5.0-8.0	-70□C (lower limit studied) 60□C (upper limit studied - survival is time dependent)	up to 5%

Since the organism has been isolated from pork products and pork processing plants (Wesley, 1997), it was decided that the addition of two more environmentally-encountered stress variables would be beneficial in the study. Sodium nitrite is commonly added to pork products for control of *C. botulinum* spores and to maintain cured meat color and texture. The legal limit for addition of sodium nitrite in sausage is 156 ppm (µg/ml). Also, sodium tripolyphosphate (STPP) is one of the phosphates commonly added to ground meat products which, in addition to increasing the moisture binding capacity, can also act as antioxidant, buffer, emulsifier and sequestrant. The recommended limit for use of STPP is up to 0.5%.

The experimental model was defined with outer limits as follows:
Temperature: 12-37□C
Initial pH: 6.0-7.5
NaCl (%): 0-3.5%
Na nitrite(µg/ml): 0-180
STPP (%): 0-0.012

Thus, a fractional factorial plus central composite type model was designed, which included:
a) one full-factorial design that tested all possible combinations of the following variables (32 combinations):
temperature: 19□ or 30□C
Initial pH: 6.45 or 7.0
% NaCl: 1 or 2.5
Sodium nitrite (µg/ml): 52 or 128

STPP(%): 0.0035 or 0.0085

b) One center point which represented midpoint values (temperature = 24.5□C, pH = 6.75, NaCl = 1.75%, Na nitrite = 90 µg/ml, STPP = 0.006%) for all five variables.

c) Ten 'star' points that tested each extreme variable values successively against midpoint values of the other four variables.

d) Controls: at 19, 24.5 and 30□C at each initial pH of 6.0, 6.45, 6.75, 7.0 and 7.5.

Two replications were done for each combination, with five replications tested for the center point combination.

Methods: A three-strain cocktail of *A. butzleri* (human strains, obtained from USDA ARS NADC, Ames, IA) was used in this study. These were chosen from a pretrial study which identified these three strains as having characteristics representative of six strains originally selected for the study. Cultures were grown in Ellinghausen McCullough Johnson Harris (EMJH) medium (Intergen Inc., Purchase, N.Y.) supplemented with 2% Oxyrase (Oxyrase Inc.), in 250 ml polystyrene tissue culture flasks incubated with gentle shaking (50 rpm) on an orbital shaker for 30 h. The cultures were centrifuged at 7000 rpm/25 min, washed twice in 0.1M phosphate buffered saline (PBS) and resuspended in PBS so that the culture density was approximately 9 log CFU/ml. Cultures were stored at 4□C until used.

Solutions of NaCl (35%), Na nitrite (1%), STPP (1%), HCl (2.25N) and NaOH (1N) were prepared in sterile distilled water, filtered through 0.2 µm cellulose nitrate filters and stored at 4□C until used. For the experimental runs, EMJH medium was combined with appropriate amounts of NaCl, Na nitrite and STPP. The pH was adjusted as necessary and 2% Oxyrase added to each 20 ml volume in individual tissue culture flasks. Equal concentration amounts of appropriate dilutions of the three strains were added, to adjust the cell density to approximately 3 $\log_{10}$ CFU/ml. Samples were withdrawn at time zero, diluted as necessary, plated on 5% bovine blood agar plates and incubated at 37□C in an CO_2 incubator with a gas atmosphere of 5% O_2, 10% CO_2 and 85% N_2 for up to 48 h. Colonies were counted as log CFU/ml and converted to $\log_{10}$ counts. Samples from each flask were subsequently withdrawn at selected intervals (based on pre-trial runs), similarly plated and counts determined, for up to 10 days.

Data obtained from the growth curve experiments was fitted to the modified Gompertz equation using nonlinear regression analysis with a Gauss Newton iteration in SAS. Fitted curves generated the Gompertz parameters A, C, M and B, which were then used to calculate the derived growth characteristics (MPDs, GTs, LPDs and EGRs) of *A. butzleri* for each combination studied.

Modified Gompertz equation: lognumber = A+C*exp(-exp(-B*(time-M))) where

lognumber = $\log_{10}$ count of bacteria ($\log_{10}$ CFU/ml)

A = initial level of bacteria (log_{10} CFU/ml)
C = number of log cycles of growth (log_{10} CFU/ml)
B = relative growth rate at M (log_{10} CFU/ml/h)
M = time at which the absolute growth rate is maximum (h)

MPD = maximum population density (log_{10} CFU/ml) = A + C
LPD = lag phase duration (h) = M-1/B
GT = generation time (h) = log_{10} 2*e/B*C
EGR = exponential growth rate (log_{10} CFU/ml/h) = B*C/e

Gompertz parameter values and derived growth parameter values were subjected to response surface regression analysis using the RSREG procedure in SAS, with temperature, initial pH, sodium chloride, sodium nitrite and sodium tripolyphosphate levels as independent variables.

Validation study: Irradiation-sterilized raw ground pork portions were mixed with appropriate amounts of curing salts (sodium chloride, sodium nitrite and sodium tripolyphosphate) and dispensed into flasks. The pH of each portion was adjusted to the desired level, the samples inoculated with a three-strain mixture of *A. butzleri* at approximately 3.0 log_{10} CFU/ml, and the flasks were incubated at selected temperatures. Inoculated control samples of ground pork (non-pH adjusted, without curing salts) were also used in the study. 10 g samples were weighed at intervals, stomached with 0.1M PBS and diluted. Aliquots were plated on 5% bovine blood agar plates which were incubated at 37°C/48 hrs in a modified gas atmosphere.

The following ranges represented variable values used in this study:

Temperature:	14-32°C
pH:	6.5-7.0
sodium chloride:	0.5-2.0%
sodium nitrite:	50-150µg/ml
sodium tripolyphosphate:	0.005-0.0075%

V. Results

An analysis of the data determined that *A. butzleri* strains were more sensitive to lower concentrations of STPP that previously described for other organisms in literature (Zaika et al, 1993), with growth occurring from 0-0.016% STPP and survival up to 0.02% STPP. An analysis of the growth data determined that maximum population densities achieved were largely independent of the five variables, with means of 8.8-9.2 log_{10} CFU/ml.

For the control runs, where temperature and initial pH were the two interacting

variables, temperature was found to be primarily responsible for LPDs. Within each temperature, pH 6.45 showed the longest LPD (Fig. 1). LPDs and GTs increased and EGRs decreased, with increasing concentrations of either Na nitrite or STPP. Increasing STPP levels were found to have a greater effect on the LPDs, GTs and EGRs as compared to Na nitrite and NaCl levels (Table 2, Fig. 2). In addition, some interaction effects were noted, between the five variables studied.

Growth data was analyzed by regression analysis using second order response surface models with temperature, initial pH, NaCl (%), phosphate (%) and nitrite(µg/ml) as independent variables. A quadratic polynomial model in terms of these 5 variables was calculated for the natural logarithm-transformed Gompertz parameters and the derived growth parameters. The general form of the regression is:

Response = $a_0 + a_1$*temp + a_2*pH + a_3*NaCl + a_4*phosp + a_5*nitrite + a_{12}*temp*pH + a_{13}*temp*NaCl + a_{14}*temp*phosp + a_{15}*temp*nitrite + a_{23}*pH*NaCl + a_{24}*pH*phosp + a_{25}*pH*nitrite + a_{34}*NaCl*phosp + a_{35}*NaCl*nitrite + a_{45}*phosp*nitrite + a_{11}*temp*temp + a_{22}*pH*pH + a_{33}*NaCl*NaCl + a_{44}*phosp*phosp + a_{55}*nitrite*nitrite

The parameter estimates i.e. a_0-a_{45} for each of the derived growth parameters are presented in Table 3. R-squared values for the response surface model for each of the derived growth parameters are as listed in Table 4. Factors significant in determining each derived growth parameter are listed in Table 5, with relevant t values, in decreasing order of importance.

Results obtained from the study of *A. butzleri* in ground pork indicate that the organism survives, but does not grow in raw ground pork, under any of the conditions studied in the validation study. Initially inoculated population levels of approximately 3 $\log_{10}$ CFU/ml fell to undetectable levels over a period of 72 h.

VI. Conclusions

The model described allows for the predictive growth response of *A. butzleri* in laboratory growth medium within the environmental parameters listed in the model design. This model would be of use in predicting the growth of this pathogen in substrates of similar nutritional and moisture content.

When raw ground pork was used as the growth substrate, the population levels of *A. butzleri*
did not increase, both in the control samples, and in samples of the variable combinations tested. All variables used in the validation study i.e., temperature, pH, sodium chloride, sodium nitrite and sodium tripolyphosphate were within the limits selected for the earlier predictive broth study. For any predictive modeling study, it is important to note that the growth data obtained under laboratory culture conditions using nutritionally-perfect media is not always an indication of the behavior and fate of the organism in real-use situations i.e., in this case, in raw ground pork. This should be considered when selecting information to be used in evaluating the microbial hazard related to this microorganism. The inability of *A.*

butzleri to grow and increase in population size in raw ground pork is a matter for some speculation. Literature reports of studies done on the related *Campylobacter* spp. indicate that the organism is highly sensitive to desiccation (ICMSF, 1996), and the results that we have obtained suggest that this may indeed be a potential cause leading to the incapacity of the organism to grow in ground pork. Thus, our studies suggest that raw ground pork is not a suitable growth substrate for this pathogen. Since the primary purpose of any microbiological safety study is to ultimately ensure the safety of the food product in question, our study on ground pork serves to demonstrate that this pathogen may survive, but does not increase in numbers in raw ground pork.

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Table 2: Effect of STPP and Na nitrite concentrations on growth parameters of *A. butzleri*

% STPP [a]	LPD(h)	GT(h)	EGR (log_{10} CFU/ml/h)
0	14.66	1.68	0.17
0.006	21.66	1.54	0.21
0.012	61.7	3.73	0.08

Na nitrite (µg/ml)[b]	LPD(h)	GT(h)	EGR(log_{10} CFU/ml/h)
0	18.97	0.86	0.34
90	21.66	1.54	0.21
180	23.25	1.96	0.15

[a] temperature = 24.5°C, pH = 6.75, NaCl = 1.75%, Na nitrite = 90 µg/ml

[b] temperature = 24.5°C, pH = 6.75, NaCl = 1.75%, STPP = 0.006%

Table 3: Parameter estimate values for natural logarithm-transformed derived growth parameter values

Parameter estimate	ln LPD	ln MPD	ln GT	ln EGR
a_0	-5.593802	-47.43149	45.8686039	-47.0692224
a_1	-0.354763	0.06726	-0.2229234	0.2229235
a_2	3.887451	15.04880	-12.5484117	12.5484117
a_3	2.817541	-4.85980	-2.8862619	2.8862619
a_4	-328.813844	793.63238	-405.7264558	405.7264564
a_5	-0.001176	-0.14473	0.1775522	-0.1775522
a_{12}	0.041105	-0.03546	-0.0097748	0.0097748
a_{13}	-0.020542	0.00161	0.0130189	-0.0130189
a_{14}	2.854623	0.24313	7.5323283	-7.5323283
a_{15}	0.000233	0.00028	-0.0008008	0.0008008
a_{23}	-0.445378	0.81110	0.4715146	-0.4715146
a_{24}	55.170670	-147.43859	11.3007842	-11.3007845
a_{25}	0.000527	0.02094	-0.0225118	0.0225118
a_{34}	-11.199723	10.18758	45.8662376	-45.8662375
a_{35}	0.000453	-0.01189	-0.0033654	0.0033654
a_{45}	-1.199373	-0.39080	1.4248667	-1.4248667
a_{11}	0.000663	0.00316	0.0043149	-0.0043149
a_{22}	-0.366401	-1.08596	0.9392360	-0.09392360
a_{33}	0.322879	-0.02986	-0.0143558	0.0143558
a_{44}	2879.725182	14840.50901	-521.9755529	521.9755517
a_{55}	-0.000001	0.00007	-0.0000310	0.0000310

Table 4: R^2-values for the response surface analysis for derived growth parameters

Parameter	**R^2- value**
ln (LPD)	0.8388
ln (EGR)	0.8357
ln (MPD)	0.6850
ln (GT)	0.8357

Table 5: t-values of five factors (independent & interacting) most significant in determining ln derived growth parameter values

ln LPD	**ln EGR**	**ln MPD**	**ln GT**
NaCl*NaCl (5.92)	nitrite (-5.72)	NaCl*nitrite (-4.70)	nitrite (5.72)
phosp*nitrite (-3.19)	pH*nitrite (5.01)	nitrite (-3.45)	pH*nitrite (-5.01)
pH*NaCl (-2.67)	temp*nitrite (3.52)	pH*nitrite (3.43)	temp*nitrite (-3.52)
Temp*NaCl (-2.45)	pH*pH (-2.83)	pH*NaCl (2.54)	pH*pH (2.83)
NaCl (2.44)	PH (2.76)	pH (2.53)	pH (-2.76)

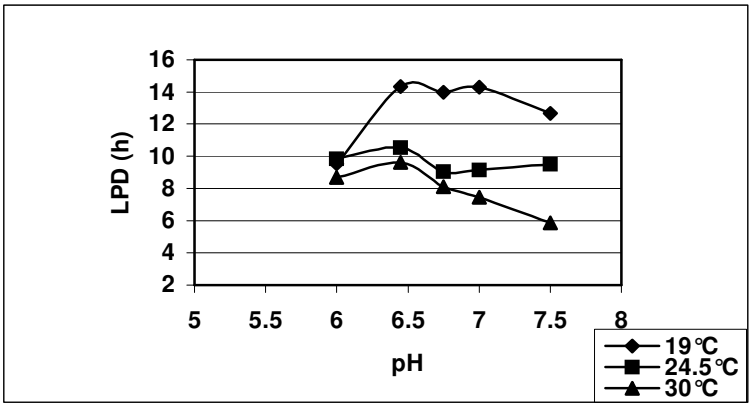

Fig 1: Effect of initial pH on LPDs of *A. butzleri* at three temperatures

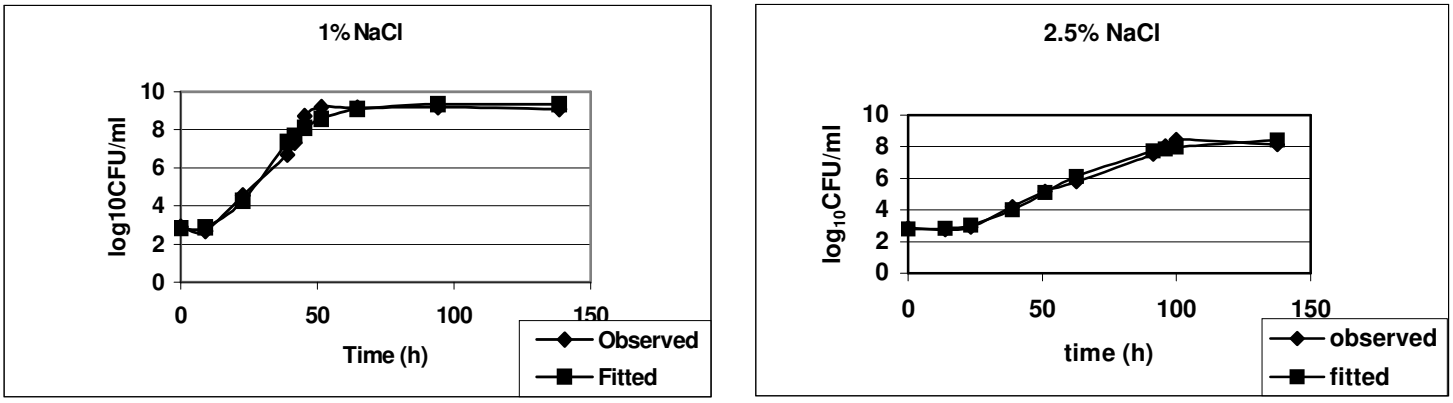

Fig 2: Effect of NaCl (1% and 2.5%) on the growth of *A. butzleri* in EMJH medium
[Other variables: temperature (19°C), pH (7.0), Na nitrite (52 µg/ml), STPP (0.0035%)]

LPD = 15.48 h
GT = 1.46 h
EGR = 0.2 $\log_{10}$ CFU/ml

LPD = 26.42 h
GT = 3.62 h
EGR = 0.08 $\log_{10}$ CFU/ml