MODULATION OF *CLOSTRIDIUM BOTULINUM* OUTGROWTH AND TOXIN PRODUCTION BY BLOOD FRACTIONS OR MODIFIED ATMOSPHERES.

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SUMMARY

Clostridium botulinum spores were evaluated for outgrowth and toxin production with added blood fractions or modified atmospheres. Dried bovine blood fractions (0-5%) were added to cured beef (156 ppm NO_2/550 ppm ascorbate), inoculated with 300 Type A&B spores/g, heated to 80°C for 20 min, then stored *in vacuo* for up to 10 wk at 27°C. ATCC 7949 (Type B, proteolytic) spores were inoculated into Hungate tubes containing broth (pH 5-7, [NaCl] 0-3%), purged with 0-6% O_2, and stored at 20°C for 60 d. Iron levels from blood fractions were inversely related to toxin lag time. Thus, blood fractions with significant levels of iron reduced the antibotulinal efficacy of $NaNO_2$. Lower pH, higher salt, and O_2 levels inhibited growth and toxin, and increased germination times. Therefore, combinations of low oxygen tension, moderate pH and salt levels inhibited *Clostridium botulinum* outgrowth and toxin development.

INTRODUCTION

Control of *Clostridium botulinum* in pasteurized and refrigerated meat products is maintained by preventing germination and outgrowth, rather than by destroying the thermally resistant spores (Hauschild, 1989). Inhibition of this anaerobic neurotoxigenic pathogen is achieved classically by limiting initial spore loads and creating unsuitable growth conditions. These include: aerobiosis, brine ≥10 %, pH < 4.6, a_w < 0.96, microbial competition, and chemical preservatives such as sodium nitrate. Recent market demands for fresh, refrigerated, convenience products have been met by the industry with the introduction of foods which are packaged under low oxygen tension after having received a combination of mild bacteriostatic treatments (Miller, 1988). Yet the potential for *Clostridium botulinum* germination, outgrowth, and toxigenesis has been insufficiently studied under these conditions. Furthermore, incorporation of iron-rich additives, such as blood, to meat products may inhibit the antibotulinal role played by sodium nitrite (Tompkin et al. 1978) in the so-called "classic" and "new generation" refrigerated foods. Therefore, the objectives of this study were to determine the 1) effects of added blood fractions on the antibotulinal efficacy of sodium nitrite in a cured beef

sausage, and 2) interactive roles of oxygen, pH, and salt on Clostridium botulinum germination, outgrowth, and toxigenesis, and

MATERIALS AND METHODS

Cultures: For blood fraction experiments a mixture of Clostridium botulinum Type A (33, 62A, 69) and Type B (999, 169, ATCC 7949) strains were used. Studies on effects of oxygen tension were conducted using the proteolytic Type B strain ATCC 7949.

Growth Media: Botulinum Assay Medium (BAM) (Huhtanen, 1979) without thioglycollate was used for broth studies. Two percent agar was included for plating medium.

Experimental design and analysis:

1. Blood fraction experiment

Beef sausages were formulated with dried bovine blood fractions including: hemoglobin (HB), plasma (PL), red blood cells (RB), and whole blood (WB). Each blood fraction was substituted for beef at levels varying between 0% and a maximum of 5% in the formulations. Additional additives included 2.5% NaCl, 156 µg/g $NaNO_2$, and 550 µg/g NaAscorbate. Five gram samples (including 300 heat shocked spores/g) were vacuum sealed in low oxygen permeable film and pasteurized at 80°C for 20 min. Samples were then double bagged under vacuum and stored for 1-10 weeks at 27°C. At weekly intervals samples were analyzed and quantified for anaerobic growth by plating dilutions onto BAM plates; nitrite levels were determined using the AOAC procedure; iron levels of meat samples were determined by atomic absorption spectroscopy according to AOAC procedures; pH measurements were made on liquid homogenates of samples after being heated (to inactivate toxin) then cooled to room temperature; mouse bioassays were used to determine the presence of botulinum toxin. Statistical analyses and curve fittings were performed using RS/1, an integrated technical analysis program.

2. Modified atmosphere experiment

BAM broth was adjusted to pH values and salt levels of 5-7 and 0-3%, respectively. Five mL portions of the adjusted broths were added to 20 mL Hungate tubes. After sterilization and anaerobic equilibration, 500 spores/mL were added. Then, tubes were equilibrated with individual gas mixtures containing either 100% nitrogen or 0.6%-2.4% oxygen with the balance being nitrogen. These environments were maintained by purging anaerobic jars containing the tubes with mixtures of the experimental gas mixtures. Oxygen levels were determined with a Systech 2550 Oxygen Analyzer. Tubes were incubated at 20°C for 90 days or until germination occurred. Germination was monitored by visual observation and turbidity measurements using spectrophotometric measurements at 610 nm. After 90

days viable spores in two remaining tubes were determined by plating dilutions on to BAM agar. Aliquots from these tubes and all tubes showing growth were assayed for toxin using an ELISA technique.

RESULTS AND DISCUSSION

1. Blood Fraction Experiment

Significant correlations ($p<0.01$) were observed between added blood fraction levels for HB, WB, and RB and measured iron in the sausage formulations. Plasma lowered by dilution endogenous iron levels in the beef (Table 1). Five percent added hemoglobin increased iron levels by ten-fold above the hemoglobin-free control. Red blood cells and whole blood increased iron levels less markedly. In all cases, except plasma, there were proportional increases between ingoing blood fraction levels and detected iron levels in the sausages. Iron levels in beef varied between 13-28 mcg/g.

Residual nitrite levels at day 0 varied between 0-40 ppm, and quickly fell to below 10 ppm, usually within 2 weeks. Similar results were observed by Kim et al. (1987). There was no apparent correlation between iron levels in the sausages and nitrite levels, nor were there trends among blood fractions. The combination of ascorbate, reductants in the beef, and the spores most likely accounted for the rapid nitrite depletion.

pH values of the sausages ranged from 5.5 to 7.9. There was no clear correlation between pH and lag time to toxin detection. While bacterial growth generally increased over time to a maximum density of about $Log_{10}=8$, the rate of increase was not correlated with either the iron level, residual nitrite, or toxin presence. The direct plating also indicated contamination, which was minimal.

Figure 1 shows the relationship between ingoing iron levels and the time until detection of toxin (lag) in cured sausages. There was a high correlation ($R^2=0.88$) between these two factors, with toxin lag time being inversely related to iron levels. An exponential decay curve gave the best predictive fit. Sodium nitrite protected the product against *Clostridium botulinum* toxigenesis for the entire 10 wk incubation period when plasma (all levels) or red blood cells (0 and 0.5%) were added. There was at least 3 wks of protection at 27°C in samples which did not include added blood fractions. Control samples, without nitrite were always toxic by 2 weeks at 27°C. A few samples where neutralized with antitoxin Types A & B; only type A toxin was produced in these samples.

Antibotulinal effects of sodium nitrite may be due to its intracellular binding to and inactivation of energy yielding sulfur-iron enzymes (Woods and Wood, 1982). Iron-containing compounds, such as hemoglobin, ferritin,

or transferrin, which are rich in blood fractions, may bind to sodium nitrite extracellular- ly, thus preventing it from entering the clostridial cell. The nitrite, therefore, cannot produce its antibotulinal effects (Tompkin et al., 1987).

2. Modified atmosphere experiment

Growth and toxin production were detected at oxygen levels between 0-2%. One percent oxygen was the maximum level where germination and toxin were detected (Fig 2). The single exception was 1 tube which grew at 2%, but was atoxigenic. At oxygen levels between 0-1% growth occurred rapidly (≤6 days) when optimum pH (7) and salt levels (0%) were maintained. There was a clear trend of diminished growth in the presence of reduced pH and increased salt. Toxin production was more sensitive to the interactions of these environmental factors than was growth.

There are a paucity of data concerning the specific oxygen tolerance for growth and toxin production in Clostridium botulinum. The results from this study demonstrate that spores will grow at oxygen levels normally encountered in vacuum and modified atmosphere packaged foods. Growth and toxigenesis can be prevented by the manipulation of other environmental factors, such as pH or salt levels.

CONCLUSIONS

1. Blood fractions containing iron, such as whole blood, hemoglobin, and red blood cells, reduced the antibotulinal efficacy of sodium nitrite.

2. Plasma can safely replace up to 5% of the beef without increasing the risk for botulinal growth and toxin production.

3. Spores of Clostridium botulinum strain C11 (type B) outgrow at oxygen levels below 2% unless other barriers are imposed, such as acidification or added NaCl.

4. There are additive or synergistic effects among oxygen tension, pH, and salt levels which can be used to prevent growth and toxigenesis of Clostridium botulinum.

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Table 1.

EFFECT OF BLOOD FRACTION SUPPLEMENTATION ON %IRON IN CURED BEEF SAUSAGES

FORTIFICATION LEVEL (%)	IRON LEVELS (ppm) PLASMA	WHOLE BLOOD	RED CELLS	HEMOGLOBIN
0.00	14.4	27.09	13.4	15.7
0.50			23.4	
0.63				37.7
1.00			43.3	
1.25				47.1
1.50	13.8	49.89		
2.00			58.5	
2.50	13.7	65.1		80.9
3.00			70.7	
3.50	12.9	83.5		
5.00	10.5	102.2		156.1
BEEF	15.9	27.75	13.3	17.21

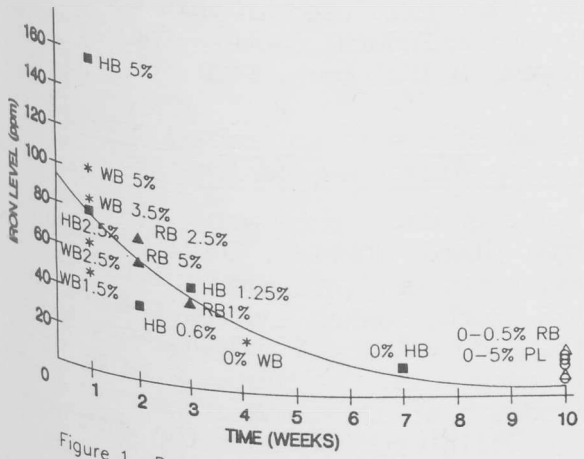

Figure 1. Relationship between iron level in beef sausage and toxin lag time ■ (Hemoglobin 1-5%) ▲ (Red Cells 0-3%) ✱ (Whole Blood 0-5%) O Plasma (0-5%) Open marks indicate that no toxin was detected.

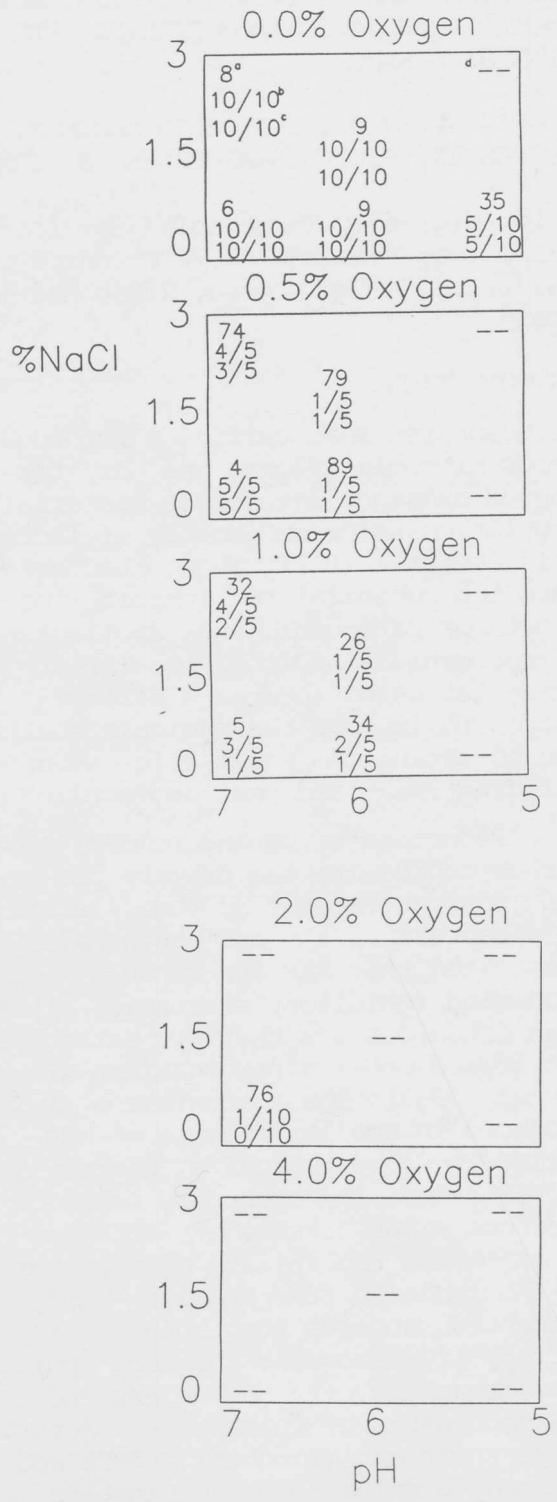

Figure 2. Effects of interactions among %oxygen, pH, and salt on germination of C. botulinum type B in BAM broth.
a) Days to germination
b)#tubes which grew/#total tubes
c)#toxigenic/#tubes
d) No growth, no toxin