ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF CLOSTRIDIA FROM "BLOWN-PACK" VACUUM PACKAGED BEEF

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Abstract – Psychrophilic *Clostridium* spp. were isolated from commercially spoiled "blown-pack", vacuum package beef. Molecular methods were used to identify these organisms. Heat resistance of psychrophilic *Clostridium* spp. endospores was investigated under aerobic and anaerobic conditions. *Clostridium* spp. endospores were suspended in saline and were heated to 70, 80 and 90°C for up to 60 min. Psychrophilic *Clostridium* spp. were able to survive heating at 80C under anaerobic conditions.

Key Words – heat resistance, psychrophilic, spores.

I. INTRODUCTION

Clostridium spp. have not traditionally been implicated as a major cause of meat spoilage. However, the causative agent for "blown-pack" spoilage of vacuum packaged meat has been identified as being *Clostridium* spp. [1-4]. "Blown-pack" spoilage is characterized by gross distention due to gas production by *Clostridium* spp. and by the production of offensive odours at refrigeration temperature within 4 to 6 wk [5]. Both *Clostridium estertheticum* and *Clostridium estertheticum* subsp. Laramie, particularly, have been implicated in "blown-pack" spoilage of beef from southern Africa, northern Europe and North America [1,2,6,7], and in vacuum packaged lamb from New Zealand [4]. *Clostridium gasigenes*, a psychrotrophic organism, has only been implicated in "blown-pack" spoilage in vacuum packaged lamb from New Zealand [8].

Recently, there have been cases of "blown-pack" spoilage of fresh beef in Canada [7, 9]. One reason why these organisms have become a problem in meat is because they persist on beef carcasses after the use of interventions. Theses interventions can include heat (steam pasteurization), UV, and chemicals (lactic acid washes). *Clostridium* spp. persist as they produce endospores that are extremely resistant to these interventions. Endospores are resistant to wet and dry heat, ultraviolet radiation, toxic chemicals, and desiccation [10]. The presence of spoilage *Clostridium* spp. on fresh beef is a continuing problem for the industry. The objectives of this study were to isolate and identify spoilage organisms from vacuum packaged beef and to investigate heat resistance of psychrophilic *Clostridium* spp.

II. MATERIALS AND METHODS

Isolation of psychrophilic Clostridium spp. from "blown-pack" beef

A spoiled vacuum packaged whole raw beef loin was inspected for leaks and stored at 4°C until further processing. A sterile silicon plug was inserted into the packaged to aid with gas removal. After the gas was removed, the meat package was transferred into the anaerobic hood. The anaerobic hood was in a cold processing room set at 10°C. The surface of the package was cleaned with 70 % ethanol before using a sterile blade to open the package. A 2 x 5 cm surface sample was obtained using a sterile blade and tweezers. The sample was transferred into a sterile stomacher bag. Meat purge was also collected for sampling. Dilutions were made in sterile 0.85% saline and samples were plated onto Reinforced Clostridial Medium agar (RCM; Difco; Becton, Dickinson and Company, MD, USA) [3] with 0.5 % glucose and 5% defibrinated sheep blood (Oxoid SR51; MT, USA). Plates were incubated at 7°C for 3 wk in an anaerobic jar. Colonies were chosen and streaked for purity on RCM blood agar and incubated anaerobically at 7°C for 3 wk.

Separation of clonal isolates by Restriction Length Polymorphism (RFLP)

The reference strains used for molecular comparison included *Cl. frigidicarnis* ATCC BAA154 and ATCC BAA155, *Cl. estertheticum* ATCC 51377, and *Cl. putrefaciens* ATCC 25786. Genomic DNA was isolated using a DNeasay Blood and Tissue Kit (Qiagen; MD, USA). The manufacturer's recommended protocol for

isolation of nucleic acid from Gram positive bacterial was followed, with modification to the lysis procedure. To improve lysis, the cells were resuspended in enzymatic lysis buffer (50 mg/mL lysozyme; Sigma, MO, USA) and incubated at 37°C for 90 min. The recommended protocol was followed for the remainder of the procedure. To analyze the 16S rDNA gene, PCR was performed using universal (eu) bacterial primers. The primer sequences were: pA(forward) 5'-AGA GTT TGA TCC TGG CTC AG-3' and pH* (reverse) 5'-AAG GAG GTG ATC CAG CCG CA-3' [11]. These primers are complementary to the conserved region of the 16S rDNA gene. The genomic DNA isolated from each culture was used as the template. PCR was completed using the modified Broda *et al*. [8] protocol.

PCR-amplified 16S rDNA of the reference (type strains) and meat strains were digested with *Alu*I, *Hha*I, and *Hae*III endonucleases (Invitrogen, CA, USA). Restriction digests contained the following: 10 μL of PCR product, 2 μL of the appropriate buffer and 10 U of restriction endonuclease and balance Milli-Q water for a total volume of 20 μL [8]. The mixtures were incubated overnight at 37 ºC. Products of the restriction digest were separated by electrophoresis in a 2.0% (w/v) agarose gel at 90 V for 1.5 h. A 1 Kb Plus DNA molecular weight marker (Invitrogen, CA, USA) was used as a size marker. The gel was stained with ethidium bromide and banding patterns were visualized using a UV transilluminator. Restriction of PCR products was replicated three times.

Identification of psychrophilic Clostridium *spp.*
Identification of the colonies was completed using 16S rDNA sequencing by PCR as described above. Sequencing of the 16 S rDNA of the isolates from RCM blood agar was done by Macrogen (MD, USA). Primers (pH* and pA) were diluted to a concentration of 1.6 pmol/μL. Sequencing with the forward and reverse primers was done separately and aligned using National Center for Biotechnology Information (NCBI) alignment tool (blastn). The sequence was compared to gene databases.

Preparation of spore suspensions for heating
The bacterial strains used in this study included: BP-1 (BP – "blown-pack" isolate), BP09-01, BP09-13, and *Cl. estertheticum* ATCC 51377. Strains were grown and allowed to sporulate anaerobically in peptone, yeast, glucose, starch (PYGS) broth [12] at 7°C for 3 months. Endospores were harvested and washed 10 times with 50 mL of cold saline by vortexing and centrifugation at 7500 x *g* for 30 min. Endospores were resuspended in 25 mL of saline and stored at 1°C until used.

Heat Resistance
Heat resistance was determined by heating in an anaerobic hood or on the bench (aerobically). Endospores were distributed (1 mL) into sterile eppendorf tubes. For heating under anaerobic conditions, tubes were transferred into an anaerobic hood and heated in a dry bath incubator (Isotemp Digital 2-Block Model 125DQ, Fisher Scientific, IA, USA). Temperature and time parameters were: 70, 80 and 90°C for 0, 4, 8, 15, 30, and 60 min. Immediately after heating, samples were put on ice. Heated endospores were diluted in pre-reduced saline and spot platted onto pre-reduced PYGS agar. Plates were transferred into an anaerobic jar and incubated at 7°C for 3 wk. Identical protocols were used to heat endospores in an aerobic environment.

III. RESULTS AND DISCUSSION

Isolation and identification of spoilage organisms
The meat, obtained from a local processor, was approximately six wk old upon receipt. The meat package exhibited gross pack distention. The exterior colour of the meat was dark and purple and the interior of the meat was pink. When exposed to oxygen, the meat turned grey. A large amount of purge was collected from the blown vacuum pack. Odours of the spoiled meat were described as "cheesy", "dairy", and "putrid". The odour of the meat and purge was offensive.

Colonies (129) were chosen based on colony morphology. Of the 129 colonies chosen, only 38 were strict anaerobes. Using RFLP, three colonal isolates were observed. These three isolates were sent for sequencing. BP-1 was identified as *Clostridium putrefaciens*. However, *Cl.*

putrefaciens generally does not grow at refrigeration temperatures, nor does it produce enough gas to cause "blown-pack" spoilage. BP-1 was able to produce gas and grow at refrigeration temperatures (results not shown) when inoculated onto meat and stored at 4°C. BP09-01 and BP09-13 were identified as *Cl. estertheticum*, which are known to cause "blown-pack" spoilage. RFLP patterns for BP09-01 and BP09-13 were different (results not shown), indicating they could be different strains.

Heat resistance

After heating under anaerobic conditions at 70°C, endospores of BP-1, BP09-01 and BP09-13 were not detected after 8 min of treatment (Figure 1). At 80°C, all strains except for BP09-13 had viable counts after 60 min of treatment (Figure 2). At 90°C, *Cl. estertheticum* ATCC 51377 had a slower rate of kill than other strains. BP09-01 was the most heat sensitive of the four strains. Heat shrinking vacuum packages of fresh meat has the potential to accelerate the appearance of "blown-pack" spoilage [13].

Endospores of all of the strains tested did not survive more than 4 min of heating under aerobic conditions at 70, 80 or 90°C (data not shown). Temperature can play a role in initiating germination of endospores. All of the *Clostridium* spp. tested may have begun to germinate after heat treatment. Anaerobic jars do take some time to become completely anaerobic, therefore the presence of residual oxygen may have overwhelmed any of the coping mechanisms. However, this does not explain how the endospores can survive steam pasteurization. The surface of the meat can reach 80 to 90°C during steam pasteurization [14].

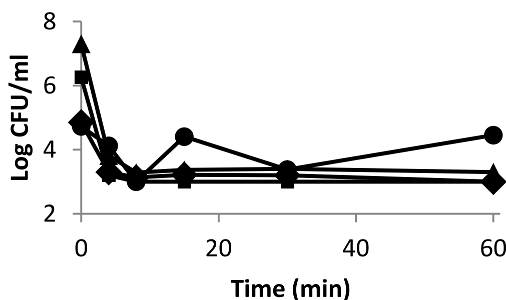

Figure 1: Log endospore counts of psychrophilic *Clostridium* spp. after heating at 70°C under anaerobic conditions with BP-1 (♦), BP09-01 (■), BP09-13 (▲), *Cl. estertheticum* ATCC 51377 (●). N=3 with a standard error of 0.74

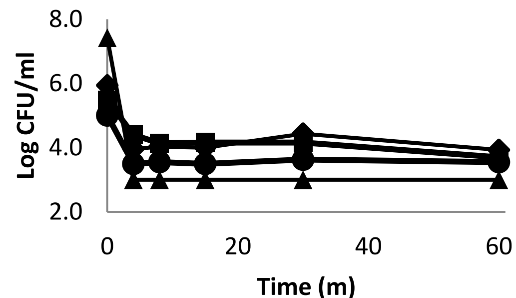

Figure 2: Log endospore counts of psychrophilic *Clostridium* spp. after heating at 80°C under anaerobic conditions with BP-1 (♦), BP09-01 (■), BP09-13 (▲), *Cl. estertheticum* ATCC 51377 (●). N=3 with a standard error of 0.89

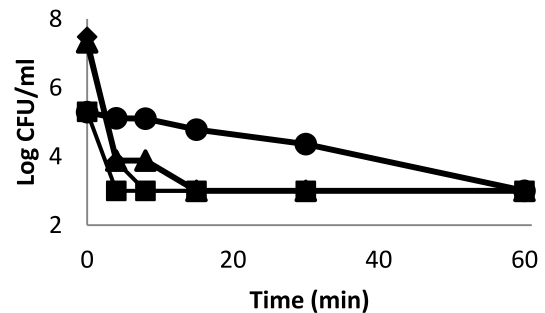

Figure 3: Log endospore counts of psychrophilic *Clostridium* spp. after heating at 90°C under anaerobic conditions with BP-1 (♦), BP09-01 (■), BP09-13 (▲), *Cl. estertheticum* ATCC 51377 (●). N=3 with a standard error of 0.77

IV. CONCLUSIONS

Psychrophilic *Clostridium* spp. isolated from blown pack spoiled fresh beef were highly sensitive to oxygen and did not survive heating under aerobic conditions. However, some strains were able to survive heating at 80°C when heated under anaerobic conditions. These *Clostridium* spp. would survive interventions used in the meat industry to reduce microbial loads on beef.

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