Spoilage of Vacuum-Packaged Refrigerated Beef by *Clostridium*

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ABSTRACT

A motile, gram-positive, spore forming, anaerobic, psychrotrophic bacterial species, probably from the genus *Clostridium*, was involved in spoilage of vacuum-packaged refrigerated fresh beef. The spoilage was associated with accumulation of large quantities of foul smelling gas and purge in the bag and loss of color and texture of the meat. Attempts to grow the organism in several laboratory media were not yet successful; however, inoculation of purge from a spoiled sample into a fresh beef, vacuum-packaging and refrigeration storage facilitated growth of this species and produced characteristic spoilage of beef.

Vacuum-packaging and refrigeration have become popular to extend shelf-life of fresh meat. The predominant bacteria associated with spoilage of vacuum-packaged refrigerated meat are several species of lactobacilli, *Leuconostoc* and *Enterobacteriaceae, Brocothrix thermosphacta* and *Alteromonas putrefaciens* (2,4,5,10,12,15). At normal pH of meat (pH 5.5 to 5.8) growth of *B. thermosphacta, A. putrefaciens* and several *Enterobacteriaceae* species are restricted (4,5,7,8), and as the storage temperature nears 0°C, the growth of psychrotrophic lactobacilli predominates (3,5,12). The predominance of psychrotrophic lactobacilli in these products can also be due to production of some antimicrobial compounds that restrict growth of associative bacteria (4,5,15). In contrast, *B. thermosphacta* and several psychrotrophic gram-negative facultative bacteria may grow in high pH beef (pH above 5.8) and cause spoilage (4-6,14,18).

The spoilage of vacuum-packaged refrigerated beef from the growth of lactobacilli, *Leuconostoc* spp. and *A. thermosphacta* is associated with sour, acid, and cheesy odor from the production of short chain fatty acids and other organic acids (2-4). In contrast, the gram-negative psychrotrophs, including *A. putrefaciens*, produce low molecular weight sulfur compounds and amines, give offensive odor and cause greening of meat (5,6,13,14,18). A specific *Lactobacillus* strain produced hydrogen sulfide with greening of vacuum-packaged refrigerated meat (16). Spoilage of vacuum-packaged meat by *Clostridium* spp. at relatively higher storage temperature has been reported (9).

At present we are investigating the microbiological causes of extensive spoilage of vacuum-packaged fresh beef produced by a large processor. The spoilage is associated with accumulation of large amounts of foul smelling gas and fluid and extensive proteolysis. Our preliminary studies suggested that the causative organism is a *Clostridium* spp.

MATERIALS AND METHODS

*Examination of commercial spoiled beef samples.* Spoiled samples of top round, chuck roll, tri tip, and strip loin with accumulation of large quantities of gas were obtained under refrigeration from the beef processor. The samples were examined for color and texture changes and fluid accumulation (purge). The bags were opened, gas was examined for odor, and the purges were used for pH and bacteriological examination and inoculation in fresh beef.

*Bacteriological examination.* The purges from spoiled samples were examined directly under a phase contrast microscope to visualize the predominant microflora and also by gram-staining and staining for spores (17) and flagella (1). The materials were serially diluted and pour plated for aerobic plate counts (plate count agar, 30°C for 2 d), psychrotrophic counts (plate count agar, 10°C for 7 d) and psychrotrophic lactic acid bacteria counts (APT agar, pH adjusted to 5.0 with lactic acid, 10°C for 7 d). For the isolation of the suspected *Clostridium* species dilutions of the purges were pour plated in thioglycollate agar (fluid thioglycollate broth + 1.5% agar), brain heart infusion agar supplemented with either 0.1% Na-thioglycollate or 0.1% Na-thiolactate + 0.05% L-cysteine, lactose egg-yolk-milk agar and tryptiticase peptone-glucose yeast extract agar (broth + 1.5% agar) (17). The plates were incubated either under vacuum or in 50:50 N₂ + CO₂ at 1 to 3°C for two to three weeks and examined for colonies. Cells from the representative colonies were purified and examined by phase contrast microscopy and gram-staining.

*Inoculation of fresh beef.* Fresh beef steaks with pH between 5.5 to 5.6 were trimmed and cut aseptically in 100 to 125 g pieces from semitendinosus muscle of cows slaughtered in our facility. Each piece was placed in an oxygen impermeable sterile plastic bag, inoculated with 1 ml of purge from a spoiled beef, vacuum sealed (about 5 mm Hg) and stored at 1 to 3°C in the dark. Controls were beef without the inoculum of purge. At intervals the samples were examined for gas accumulation, changes in color, texture and odor and the predominant microflora (by phase
RESULTS AND DISCUSSION

The spoiled commercial samples (pH 5.0 to 6.0) showed accumulation of large quantities of gas within 2 weeks and sometimes resulting in rupture of the bags (Fig. 1A). In our studies the inoculated samples accumulated gas in one week at 1 to 3°C (Fig. 1B) but the control samples did not show any gas even after 12 weeks. The gas and the meat from the spoiled samples had an offensive foul odor resembling hydrogen sulfide and the gas turned lead acetate solution black. The spoiled samples showed accumulation of large quantities of purge. The meat and the purge were initially bright pinkish-red and then changed to greenish color after about 10 to 12 weeks of refrigeration storage. The pinkish-red color could be from reactions(s) of myoglobin with metabolite(s) produced by the spoilage bacteria or due to low red-ox potential. The greenish color was due to formation of sulfmetmyoglobin from the reaction between hydrogen sulfide and myoglobin (13). The spoiled beef also had very soft texture. This texture loss and purge accumulation could be from proteolysis of muscle tissue by the proteolytic enzyme(s) of this organism. Microscopic examination of the purges from the spoiled samples, as compared to the controls, showed large quantities of myofibrils.

Phase contrast microscopy of the purges from both commercial and inoculated spoiled beef showed predominantly the presence of motile, thick rods of about 5 to 10 µm in length. Most cells were single, but some were in chains of two to three cells. The cells showed straight and tumbling movement. Materials from spoiled beef that were stored at 1 to 3°C for 8 weeks or longer had both vegetative cells and cells bearing single spore. The cells were gram-positive while the spores were terminal, large and oval (Fig. 2A and 2B). Because of the presence of tissue materials flagellar staining was not very clear; but cells with peritrichous flagella were observed. The purges from commercial spoiled samples had /ml, about 1 x 10⁶ aerobic plate count, 5 x 10⁷ psychrotrophic counts and 7 x 10⁷ psychrotrophic lactic acid bacteria counts. Representative purified colonies from the plates were biochemically identified as Leuconostoc spp. In all four agar media, used for the isolation of Clostridium spp., only Leuconostoc spp. formed colonies. Inoculation of the purge also in several broth media (trypticase peptone glucose yeast extract broth; brain heart infusion broth supplemented with 0.1% Na-thyoglycollate; meat broth containing 10% boiled meat juice, 0.5% yeast extract, 0.1% peptone and 1.0% glucose) in tubes and incubating the tubes in vacuum or sealing the broth with a layer of paraffin wax and incubating at 1 to 3°C for 3 to 4 weeks facilitated growth predominantly of Leuconostoc spp. The only way we have been able to grow the motile rods, that were present so abundantly in the spoiled samples, was by inoculating some purge from a spoiled sample into fresh beef and storing it underground at 1 to 3°C. Inoculation studies both with unheated vegetative cells and with heat treated spores in fresh beef indicated that this species was capable of sporulation as well as germination and outgrow at 1 to 3°C.

The psychrotrophic, gram-positive, motile, anaerobic, spore forming, rod shaped bacteria, probably a Clostridium spp., associated with spoilage of vacuum-packaged refrigerated beef, could not yet be grown and purified in laboratory media. We plan to heat treat spores from purge and isolate the species in suitable agar or broth for its characterization.

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