Anaerobic Sporeforming Microorganisms in Dairy Products

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INTRODUCTION

The bacterial endospore is resistant to many adverse conditions including chemicals, desiccation, irradiation, and heat. Since anaerobic sporeformers grow without oxygen and produce extremely heat resistant spores, thermal processes for canned food products often are designed specifically to inactivate these microorganisms. Anaerobic sporeformers do not create problems in the dairy industry of the same magnitude as those in the canning industry; however, the importance of anaerobic sporeformers in dairy products must not be overlooked.

Taxonomy

Anaerobic sporeformers are members of the family Bacillaceae and fall into either the genus Clostridium (gram-positive) or Desulfotomaculum (gram-negative). In general, while these organisms will not grow in the presence of oxygen, they are not as oxygen-labile as anaerobes such as Bacteroides, which will be inactivated at the slightest contact with oxygen.

In the genus Clostridium, the species butyricum, pasteurianum, tyrobutyricum, and sporogenes and others often are associated with spoilage problems in cheese. These four species have an optimum growth temperature of 37°C and will grow in an approximate temperature range of 20 to 24°C. They all produce acetic and butyric acid, and spoilage due to these organisms is called a butyric fermentation. Other important species include (a) C. botulinum, a well known producer of the toxin-causing botulism with psychrotrophic as well as mesophilic strains; (b) C. perfringens, a species with an extremely wide growth range of 20 to 50°C and a pathogen that can cause infections and food poisoning in man; (c) C. thermosaccharolyticum, a thermophile with an optimum growth temperature of 55°C.

The other genus containing anaerobic sporeformers is Desulfotomaculum. These organisms formerly were classified as clostridia; however, they use reducible sulfur compounds as terminal electron acceptors and were classified as this new genus. Examples include D. nigricans, which is a thermophile, and D. ruminis and D. orientis, which are mesophiles. D. nigricans caused sulfur stinker spoilage in canned products (7).

Spore Cycle

All spores, regardless of genus, have a keratin-like outer coat, a cortex, and an inner core or protoplast containing genetic material. The cortex appears to be responsible for maintaining the core in a dehydrated, or resistant, state (30). When conditions are appropriate, vegetative cells produce dormant spores and the mother cells lyse. Dormant spores generally must be activated prior to germination and subsequent outgrowth to a vegetative cell. Figure 1 diagrams these steps called the spore cycle. To control a spore-forming microorganism, the spores themselves can be inactivated, the vegetative cell growth can be controlled, or germination and outgrowth must be prevented. If germinated spores are held at conditions that do not support outgrowth, they will autosterilize, or become inactivated. Sporeformers do not need to sporulate; i.e., sporeformers can remain in the vegetative state indefinitely. In addition, sporulation is a one-to-one event. It is not a way of reproducing and increasing numbers of cellular units.

General Control Measures

Some general control measures for anaerobic spores are listed (Table 1). Since many, if not
most, dairy products are stored at refrigeration temperatures, or at least below room temperature, and the optimum temperature for growth of anaerobic sporeformers often is 37°C or higher, the use of low temperatures is a significant way of controlling anaerobic sporeformer growth. The lower pH associated with fermented dairy products helps inhibit growth as does the lowered water activity, or \( a_w \), found in some cheeses. In addition, preservatives such as nisin and nitrate can be effective, as can inactivation of spores through a thermal process or by using \( \text{H}_2\text{O}_2 \). Finally, good sanitation can help keep spores out of milk, and centrifugation processes have been used to remove spores already in milk and reduce potential problems.

**Toxins and Disease**

The most feared form of food poisoning is botulism, and dairy products can provide a suitable growth medium. Read et al. (33) have demonstrated that \( C.\ botulinum \) type E can produce toxin in sterilized milk at temperatures as low as 7.2°C after 12 wk of incubation and within 3 to 5 days at 30°C. In fluid milk this would not be a problem, since normal spoilage would occur before toxin production; however, in an ultra-high-temperature (UHT) pasteurized product designed to be shelf-stable at refrigeration temperatures, toxin production could occur if viable spores were present.

In processed cheese, Karim and Grecz (21) have demonstrated that both \( C.\ botulinum \) spores and toxin can be stable for long times under refrigerated conditions. Kautter et al. (23) demonstrated that \( C.\ botulinum \) types A and B can grow and produce toxin in processed cheese considered shelf-stable at room temperatures. This type of cheese is not processed as a low-acid food; hence, the potential for botulism exists. This is a potential problem only since Kautter et al. (23) added \( C.\ botulinum \) spores to cheese after the heat process used to prepare and package the cheese.

Food poisoning by \( C.\ perfringens \) has been associated most often with meats or gravies; however, \( C.\ perfringens \) spores are in milk. It has a wide growth temperature range (20 to 50°C) and could grow in mishandled milk or cheese. Hoch et al. (16) report that between 1960 and 1973 two cases of \( C.\ perfringens \) food poisoning due to dairy products involving 72 people occurred in Hungary.

The last form of disease generally could be described as clostridial infections of the infant gut. Howard et al. (17) described an outbreak of necrotizing enterocolitis caused by \( C.\ butyricum \). The latter organism is often in milk and has caused gas defects in cheese. The organism apparently was introduced to the infants through artificial feeds. This outbreak, combined with the infant botulism described by Arnon et al. (1) — although infant botulism has never been associated with the use of a dairy product — emphasizes the importance of keeping spores out of dairy products intended for use by infants. As the methodology for the culture of anaerobes improves, and workers begin looking more and more for anaerobes as the cause of diseases, the presence of anaerobic spores in dairy products may come to represent more than just a spoilage problem.

**Milk**

Pasteurized milk would not be a product affected by anaerobic sporeformers unless a psychrotroph was involved. Bhadsavle et al. (5) have demonstrated that \( C.\ bastiforme \)

### Table 1. Examples of control measures for spores.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>3.3°C</td>
</tr>
<tr>
<td>pH</td>
<td>4.6</td>
</tr>
<tr>
<td>( a_w )</td>
<td>.93</td>
</tr>
<tr>
<td>Preservatives</td>
<td>nisin, nitrate</td>
</tr>
<tr>
<td>Inactivation</td>
<td>heat, ( \text{H}_2\text{O}_2 )</td>
</tr>
<tr>
<td>Removal</td>
<td>sanitation, bactofugation</td>
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was able to spoil commercially pasteurized milk stored at 7.2°C within 46 days. Dried milk also can contain anaerobic spores, since the drying process does not inactivate spores in the fluid milk; instead, dry spores ($a_w = .35$) are more stable and will be more resistant to adverse conditions than spores of a higher $a_w$. Specifications may be needed for anaerobic sporeformers in dried milk used in formulation of other food products. Sweetened condensed milk, having a higher solids content than normal fluid milk, has an $a_w$ low enough to affect microbiological growth (36). Because of the lowered $a_w$, yeasts would be the probable spoilage organisms instead of bacteria (36), even though the pasteurization process the product receives would not inactivate all anaerobic sporeformers. Evaporated milk has a final sterilization process, as does sterilized fluid milk. In either case, anaerobic sporeformers should not be a problem unless the thermal process was inadequate or there was postprocessing contamination. Spoilage by any of the mesophilic clostridia (e.g., *C. sporogenes*) would indicate possible *C. botulinum* problems and signal alarm. Spoilage by thermophilic anaerobes, such as *Clostridium thermosaccharolyticum* or *Desulfotomaculum nigrificans*, is possible since thermal processes usually are not designed to inactivate the extremely heat-resistant thermophiles; however, elevated storage temperatures would be required to produce spoilage.

**Cheese**

The majority of work concerning anaerobic sporeformers in dairy products has been related to cheese. The primary problem is a defect caused by the gas formed by clostridia, particularly *Clostridium tyrobutyricum*. This is called "blowing," or "late blowing," since the defect often does not show up until 2 or 3 wk after manufacture, which distinguishes it from coliform gas, which occurs within 1 to 2 days of manufacture. Because butyric acid, H$_2$S, and putrefactive end products are produced by the clostridia, obvious flavor and odor defects can occur in addition to the gas defect.

**Spores in Milk**

Anaerobic spores, particularly spores of *C. tyrobutyricum*, are in the soil, in bovine feces, and in silage (13, 26, 34, 37). Numerous studies have shown that the kind of silage and spore levels it contains can affect spore levels in milk. Since the spores most often enter the milk through fecal contamination, adequate sanitation is of primary importance in controlling spores in milk. In France, Henry (15) recommends payment for milk on spore counts, 780/liter being considered adequate for anaerobic spores in raw milk. It appears that milk should contain no more than 1 anaerobic spore/ml (1000/liter).

Milk containing high levels of spores can be modified through a continuous centrifugation process called bactofugation to reduce spore levels. The temperature of the milk during centrifugation appears to affect removal, higher temperatures yielding fewer spores. Temperatures of the 60 to 80°C range appear most effective (3, 4, 27, 28).

**Inactivation**

Inactivation of anaerobic spores by heat generally requires temperatures in excess of 100°C (31). Spores from *C. sporogenes* or PA 3679 are considered the most heat resistant formed by mesophilic anaerobes. These spores have a $D_{121°C}$ of .1 to 1.5 min, depending on the conditions of spore formation (18). *C. tyrobutyricum* and other sporeformers causing late blowing in cheese have a $D_{120°C}$ of approximately .1 min, or perhaps less, again depending on the sporulation situation. Temperatures of less than 100°C will serve to activate spores, not inactivate them, allowing germination to begin when conditions are favorable. Milk is perhaps the most protective medium for heating spores. Other factors that affect heat resistance and that should be considered are the pH (lower pH decreases heat resistance), $a_w$ (dry spores are more heat resistant — fats and solids will lower the $a_w$ and provide a protective effect), and salt content.

Hydrogen peroxide can be used to inactivate spores. When treated with a 15% solution of H$_2$O$_2$ (pH 2.0, 80°C), the most resistant *Bacillus subtilis* spores had a D of 54 s (9). Other workers effectively have reduced aerobic and anaerobic spore loads with .033 to .05% H$_2$O$_2$ and 52 to 72°C (6). Catalase can be added to eliminate any excess or residual H$_2$O$_2$.

Casalis et al. (8) describe a combination of IR (3000 nm) and UV (253.7 nm) at 80°C
temperatures that reduced population of *C. tyrobutyricum* spores by 95%.

**Antimicrobial Controls**

Use of antibiotics to control sporeformers has centered on the use of nisin. Nisin can be added to cheese milk at approximately 100 to 150 Reading Units to effect control of anaerobic sporeformers (2, 20). Since the antibiotic is produced by *Streptococcus lactis*, it is possible to use starter cultures that naturally produce this antibiotic (22). In addition to being used alone, nisin has been used in combination with heat or H$_2$O$_2$ to obtain a synergistic effect and to control sporeformers, apparently inhibiting outgrowth (14).

Control of spoilage organisms by starter cultures also may occur through competitive inhibition. The dominance of starter cultures such as *Lactobacillus* (19) and *Streptococcus* (10) species at the expense of anaerobic sporeformers and other spoilage or pathogenic organisms can be due to several mechanisms. Daly et al. (10) discussed the role of *S. diacetilactis* in inhibiting pathogens, and among mechanisms suggested are antibiotic production, H$_2$O$_2$ production, nutrient depletion, and pH alteration, all of which could affect the growth of anaerobic sporeformers.

An extremely interesting area is the production of bacteriocins by starter cultures. Bacteriocins are antimicrobial particles produced by some bacteria, including *Lactobacillus* and *Streptococcus* sp. Bacteriocins are often species specific and appear to have mechanisms of action different from antibiotics. A single "hit" by a bacteriocin is apparently lethal. Bacteriocins have been associated with plasmids, and it appears possible to engineer a starter culture genetically with the capacity to produce a bacteriocin against *C. tyrobutyricum*. Maleszewski and Stec (29) suggest that bacteriocins may be a factor in the dominance of enterococci in foods. Tagg et al. (35) have written an excellent review concerning bacteriocins in gram-positive organisms.

Another area of antimicrobial control is the vaccination of cattle to produce antibodies against specific microorganisms. Korhonen et al. (24, 25) report significant antibodies to *C. tyrobutyricum* in milk of inoculated cows. These antibodies were considered of practical significance in controlling spoilage by *C. tyrobutyricum* in cheese made from milk containing these antibodies.

**Chemical Preservatives**

In Europe, nitrate, at approximately 200 ppm, routinely is used to inhibit gas defects in cheese from anaerobic spores. For many reasons, not the least of which is nitrate-nitrite controversy, nitrate probably will never be used in the manufacture of US cheese. Late blowing is a much more serious problem in Europe, possibly because of less effective sanitation procedures.

Sorbate, or sorbic acid, also can be used to control anaerobic sporeformers; .075% has been effective (38). More sorbate, in conjunction with the low pH and high salt content in cheese, also might prove effective (38). These are much higher than the normal salt in water content of approximately 4% in a cheddar cheese. However, at the pH normally found in cheese, 2.6% salt provides some inhibitory effect on anaerobic sporeformers (32).

**Fabricated or New Products**

The development of fabricated products, or products designed to imitate or replace dairy products, may lead to spoilage problems not encountered in traditional dairy products. A good example is infant soy formulas. Work in our laboratory at the University of Minnesota has shown that *Desulfotomaculum nigrificans* can exhibit extraordinary heat resistance and, hence, provide an unexpected form of spoilage (11). For this organism the key to growth is a nutritionally complete infant formula whose raw ingredients provide the source of spores (12). Both casein and soy-based infant formulas have shown occasional "sulfur stinker" spoilage by *D. nigrificans*, a type of spoilage not normally encountered in dairy products.

Unexpected problems from anaerobic sporeformers may be observed as other fabricated products are developed, particularly if they receive a thermal process and are canned. In addition, traditional products also may experience unexpected spoilage as lower fat, lower salt, or soy-protein containing products are developed to meet consumer demands.

**CONCLUSION**

A number of anaerobic sporeformers im-
important for spoilage and disease have been discussed. Potential future problems caused by anaerobic sporeformers include infant diseases, possible botulism in shelf-stable processed products, and unexpected spoilage of new products. There is a need for continued and expanded research concerning anaerobic sporeformers, particularly since chemical controls are often seen as threats by consumers, and the regulatory agencies continually are reviewing data on additives and processes.

REFERENCES


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