EMERGING THERMODURIC / THERMOPHILLIC BACTERIA AND THEIR INACTIVATION BY ULTRASONICATION

Sanjeev Anand
Associate Professor
Dairy Science Department
South Dakota State University
OUTLINE OF THIS TALK

- Common terms related to heat resistant bacteria
- Emerging thermoduric spore formers in the dairy manufacturing environment
- Thermoduric progression during Cheddar cheese manufacture – A case study
- Role ultrasonication can play to inactivate thermoduric sporeformers and spores- A case study
Some Terms
THERMODURIC BACTERIA

- Organisms that survive thermal processing treatments such as pasteurization
- The common genera considered thermoduric
  - *Bacillus*
  - *Geobacillus*
  - *Clostridium*
- More recently
  - *Paenibacillus*
  - *Anoxybacillus*
THERMODURICS THAT DO NOT SPORULATE

- Lactobacillus
- Streptococcus
- Enterococcus
- Alcaligenes
- Micrococcus
- Microbacterium
- Coryneforms
- Arthrobacter
THERMOPHILES

- Organisms that tolerate and actively grow at high temperatures
  - 45°C to 122°C / 113°F to 252°F
  - Optimum growth for most at 55°C / 131°F
  - Thermodurics may or may not be thermophilic
**Thermophiles of Concern to the Dairy Industry**

- *Anoxybacillus flavithermus*
- *Geobacillus steroothermophilus*
- *Bacillus licheniformis*
- *Bacillus coagulans*
- *Bacillus subtilis*
- *Bacillus sporothermodurans*

- Ideally grow where temperature reaches 40 to 65°C
  - Regeneration section of heat exchangers and in evaporators
  - Result in high numbers (up to $10^6\text{ cfu/g}$) in the final product

*(Scott et al, 2007; Burgess et al, 2010, Tabit and Buys, 2010)*
WHY THERMODURIC THERMOPHILES ARE DIFFICULT TO ELIMINATE?

- Form spores resistant to heat and chemicals
- Wide temperature growth range
- Exhibit a fast growth rate
  - generation time 15-20 min
- Readily form biofilms
  - Both spores and vegetative cells can attach to stainless steel and fouled surfaces
  - Foulant or biofilms may protect spores and vegetative cells against CIP chemicals

Characteristics
**BACILLUS SPOROTHERMODOURANS**

- First detected as a high heat–resistant spore (HHRS) in UHT milk
  - $D_{140}$ and $Z$ values of spores of 3.4–7.9 s and 13.1–4.2°C, respectively
- Strictly aerobic, can hydrolyze casein
- Contributes to the reduced quality of commercially sterilized milk and milk products
  - Due survival and out growth of the spores to unacceptably high levels
- Non pathogenic

**Bacillus licheniformis**

- Facultative anaerobe of *Bacillus subtilis* group
  - Other members *B. subtilis*, and *B. pumilus*
- Optimum growth temperature 30°C
  - Can grow from 15 to 55°C
- Considered a common spoilage organism isolated from raw milk
  - Some strains have been associated with toxin production

*(Mansour et al, 1999; Salkinoja-Salonen et al, 1999; Burgess at al, 2010)*
**Bacillus coagulans**

- A lactic acid forming species within genus *Bacillus*
  - Initially considered a spore-forming *Lactobacillus*
- Facultative anaerobic sporeformer
  - Some strains do not sporulate readily
  - Optimum growth temperature 40 and 57°C
  - Growth range 15°C-61°C
- Involved in coagulation and flat souring of evaporated canned milk

(Nakamura et al, 1998; Vecchi and Drago, 2006; Burgess et al, 2010; Vercammen et al, 2011)
**Geobacillus (Bacillus)**

**Stearothermophilus**

- Aerobic or facultative anaerobic endospore-forming rods
- Optimum growth temperatures is 50 °C
  - Grows between 37°C to as high as 76°C
  - Spores are extremely heat resistance with $D_{121}$ value of 42s
- Typically responsible for the flat-sour spoilage of low-acid canned foods, including evaporated milk

*(Head et al, 2008; Nazina et al, 2001; Dogan et al, 2009; Burgess et al, 2009; Viedma et al, 2010)*
ANOXYBACILLUS FLAVITHERMUS

- Obligate anaerobic or facultative aerobic
  - Optimum growth temperature is 60 and 65°C under aerobic and anaerobic conditions, respectively
  - Spores are very highly heat resistant
- Recognized as a major contaminant in milk powder
  - Normally present at low levels in raw milk
  - May reach up to $10^5\text{cfu/g}$ levels in the final product after 15–20h of plant operation
  - Being thermophilic, rapidly form biofilms

**PAENIBACILLUS SPECIES**

- Recognized as a new genus *Paenibacillus* in 1993
  - Since then, over 26 species have been identified within the genus *Paenibacillus*
- Facultative anaerobic or strictly aerobic
- Can grow from 10 to 40°C, the optimum being 37°C
- Recognized as an important fluid milk spoilage organism
  - Found in both raw and pasteurized milk

*(Daane at al, 2002; Huck at al, 2007)*
SUMMARIZING

- Several organisms represent thermodurics
- Thermodurics may or may not be sporeformers
- Spores, as well as, vegetative cells have thermal resistance
- Thermodurics may be mesophiles, thermophiles or psychrotrophs
Thermoduric progression during Cheddar cheese manufacture – A case study
A STUDY UNDER PROGRESS IN OUR LAB

- Commercial Cheddar cheese manufacturing cycles scanned
  - Samples of milk, cheese, whey, and WPC collected
- Microbial analysis for
  - Standard Plate Counts (SPC) on plate count agar at 35°C for 24h
  - Thermoduric mesophiles (TM)
  - Thermoduric thermophiles (TTh)

(Khilendra and Anand, 2013; unpublished)
Main Findings
Raw Milk Counts

- **Log cfu/mL**
- **Start**
- **Mid Day**
- **End (22h)**

Legend:
- **SPC**
- **TM**
- **TTh**
Thermoduric Thermophiles increase during long hours (22-24h) of milk pasteurization (log cfu / mL)

- Thermoduric mesophiles
- Thermoduric thermophiles
Cheddar cheese counts (2 days old)

- **Start**
- **Pre mid-day wash**
- **Post mid-day wash**
- **End**

Log cfu/g

- SPC
- TM
- TTh
Cheddar cheese counts (30 days old)

- **Log cfu/g**
- **Start**
- **Pre mid-day wash**
- **Post mid-day wash**
- **End**

Colors:
- **TTh**
- **TM**
THERMODURICS MAY CAUSE SLITS

- Causative organisms for these slits in Cheddar cheese samples
  - *Lactobacillus* spp.
  - *Clostridium* spp.
Counts during whey processing
(at the end of 22h continuous cycle)
SUMMARIZING

- Thermoduric thermophiles counts in the pasteurized milk increased with the run time
  - Mid-day wash helped to some extent in reducing their counts
  - Post mid-day run resulted in a greater build up of thermoduric thermophiles
- Post mid-day cheese samples also showed higher thermoduric thermophiles
- Thermoduric mesophiles did not show significant variations during the entire run
- Thermoduric counts of whey concentrates were below log 2.0 even at the end of a 22h long run
- Ripening studies are in progress
Inactivation of thermotolerant by Ultrasonication – A case study
A Study recently completed in our lab

- Previously reported thermoresistant strains of *Bacillus*, *Geobacillus*, and *Anoxybacillus* selected
- Propagated as vegetative cells
- Spores prepared and harvested
- Inactivation study
  - Ultrasonication parameters standardized in skim milk
    - Low frequency (20-100kHz)
    - 500 W, 20 kHz, VC 505 (Sonics & Materials, Inc., Newtown, CT, USA)
  - Ultrasonication followed by pasteurization

*(Som and Anand, 2012; unpublished)*
Main Findings
INACTIVATION OF THERMODURIC VEGETATIVE CELLS BY ULTRASONICATION

![Graph showing log reductions of spore inactivation by ultrasonication and pasteurization for Anoxybacillus flavithermus and Bacillus coagulans.](image)

- **Anoxybacillus flavithermus**
- **Bacillus coagulans**

Log reductions

- **Pasteurization**
- **Ultrasonication**
- **Ultrasonication + Pasteurization**

**Note:** U.S. Dairy Industry Spore Seminar, 02/20/2013.
INACTIVATION OF THERMODURIC SPORES BY ULTRASONICATION

% reductions in spore counts

Ultrasonication  Ultrasonication + Pasteurization  Ultrasonication + higher heat

B. licheniformis  B. coagulans  G. sterothermophilus

02/20/2013  U.S. Dairy Industry Spore Seminar
No structural changes in spores were observed after ultrasonication alone or a combined ultrasonication and pasteurization.

SEM images of B. licheniformis spores
SUMMARIZING

- Thermodurics such as *A. flavithermus* and *B. coagulans* can be inactivated to a higher extent by ultrasonication and it’s combination with pasteurization.

- Pasteurization followed by ultrasonication can effectively inactivate thermoduric spores up to 65.7% in the case of *G. stearothermophilus* spores.
  - A combination of higher heat treatment at 80°C for 1 min and ultrasonication can further enhance the inactivation to 75.3%.
ON-GOING RESEARCH

- This prelim data on the effectiveness of ultrasonication was generated during a Discovery project.
- We are currently engaged in testing the combination of ultrasonication and pasteurization in reducing counts of thermoduric bacteria and spores in non-fat dried milk.
  - An on-line ultrasonicator (1000 watts) with a frequency of 20kHz is being procured from Hielscher USA, Inc.
- The process has the potential to also improve the microbial quality of other dairy products such as fluid milk, and cheese.
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