CRITICAL PARAMETERS FOR THE PRODUCTION OF SAFE SOYBEAN TEMPEH

A Thesis

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Master of Science

by

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ABSTRACT

Acidification with lactic and acetic acid was used to test growth of *B. cereus* in soybean tempeh. The tempeh was made using two different molds as starter culture. Inoculation with *Rhizopus oligosporus* or *R. oryzae* in the soybeans acidified with lactic acid produced acceptable tempeh with *B. cereus* counts below 1×10^2 CFU g^{-1}. Adding acetic acid produced tempeh of varying quality and did not inhibit bacterial growth. Additionally, bacterial counts in the tempeh acidified with acetic acid were significantly higher ($P < 0.05$) when the soybeans were inoculated with *R. oryzae* than when inoculated with *R. oligosporus.*

BIOGRAPHICAL SKETCH

Carmen was born in Terre Haute, Indiana to Marvin, Sr. and Jenifer Wickware. She obtained her Bachelor of Science degree in Food Science from Purdue University in West Lafayette, Indiana in 2012.

To JCS, I will love you forever.

ACKNOWLEDGMENTS

I would like to thank the chair of my committee, Dr. Randy Worobo, for being so understanding throughout my time at Cornell. Thanks to John Churey and Dr. Dave Manns for their extreme willingness and patience when answering questions. To Abby Snyder, thank you for being a great friend who also let me play with your adorable puppy and Claudia Pazlopez, Giselle Guron, and Diane Schmitt for the wonderfully distracting game nights. To all faculty, staff, and graduate students at the Geneva Experiment Station, thank you for making NYSAES a community that was worth being a part of. For my friends in Ithaca, thank you for making my trips to Ithaca more than just class and doctor's appointments.

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CHAPTER 1

LITERATURE REVIEW

Unpasteurized Tempeh Outbreak

An outbreak of *Salmonella enterica* ser. Paratyphi B var. L(+) tartrate(+) occurred from consumption of tempeh that was unpasteurized and consumed raw (uncooked). The investigation of the outbreak, conducted by Griese *et al.*, showed that *Salmonella* was introduced from the tempeh starter culture, *Rhizopus oryzae.* This species is a slower growing mold than the commonly used *Rhizopus oligosporus.* This case is also unique because the tempeh manufacturer had changed starter culture vendors approximately one month prior to the date of outbreak onset (Figure 1).

In total, 89 cases were identified. The North Carolina Division of Public Health received 18 reports of *Salmonella enterica* ser. Paratyphi B var. L(+) tartrate(+) that were laboratory confirmed or linked. The 15 confirmed isolates were indistinguishable by their PFGE pattern, which had not been recorded previously in the PulseNet database (www.cdc.gov/pulsenet).

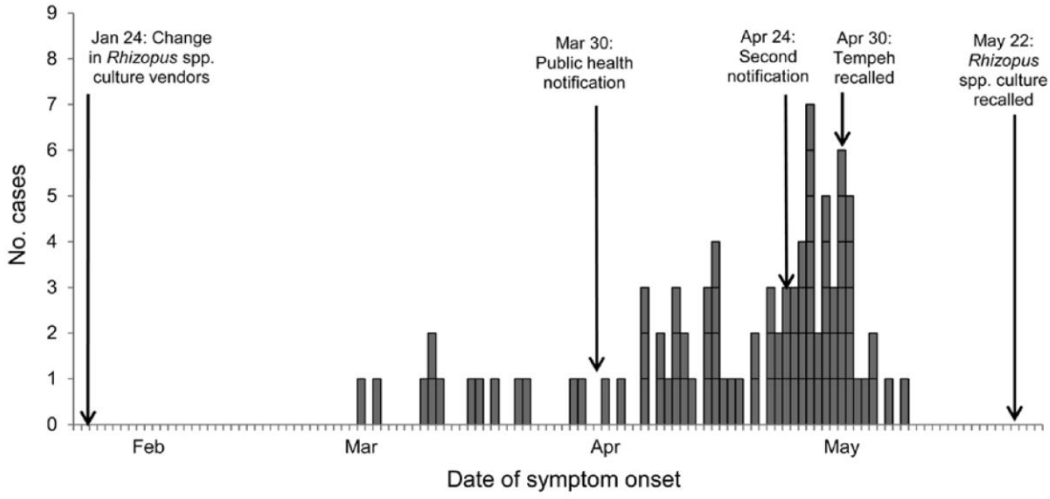

Figure 1. Cases of *Salmonella enterica* serovar Paratyphi B variant L(+) tartrate(+) gastroenteritis, by date of symptom onset, North Carolina, USA February 29-May8, 2012 (from *Griese et al.,* 2013).

Of the 89 cases, 50 individuals were selected to complete a specific questionnaire. Of those 50 who were sent questionnaires, 41 were completed and returned. From the 41 patients who returned a completed questionnaire, 18 had eaten tempeh and two of the 18 handled it regularly at a restaurant. There were three restaurants identified most frequently from the questionnaire. Managerial staffs were interviewed and food preparation was observed at the three restaurants.

After visiting the restaurants, it was found that the uncooked tempeh was prepared on the same surfaces as ready-to-eat (RTE) foods, suggesting the possibility of cross contamination and spread of the illness to people who did not consume the tempeh directly. Additional inadequate food-handling procedures included a lack of hand washing between handling raw and RTE food and bare-hand contact with RTE foods.

The North Carolina Department of Agriculture and Consumers Services informed the NCDPH that Brand A tempeh was the presumptive outbreak source approximately one month after the initial reports of illness. The company had produced unpasteurized tempeh that was sold to several restaurants in North Carolina, as well as grocery stores and a cafeteria in the southeastern US. All cases reported had some relationship in Buncombe County where the manufacturing of the tempeh occurred.

In response to the outbreak, Brand A voluntarily recalled the tempeh on April 30, a month after the first public health notification. The *Rhizopus* spp. culture was recalled a few weeks later on May 22, 2012.

Bacillus cereus *in food*

Bacillus cereus is Gram positive, endospore forming bacterium of food safety importance, particularly in regards to grains and cereals. It can be found readily in the

soil, and therefore on crops that have any contact with soil. Most strains can ferment glucose and fructose and hydrolyze starch, casein, and gelatin. The generation (doubling) time of the vegetative cells is about 30 min, but varies depending on nutrient source (Kramer & Gilbert, 1989). This doubling time is quick enough to allow a few cells of *B. cereus* to reach harmful levels in temperature abused foods such as rice.

The endospores of *B. cereus* are formed under aerobic conditions and have a greater resistance to heat when compared to vegetative cells (Kramer & Gilbert, 1989). This means that the basic thermal processes used to inactivate vegetative bacteria may be insufficient for reduction of *B. cereus* endospores, which may later germinate under permissive conditions.

Consequently, proper food handling in relation to temperature control is a major factor in the prevention of *Bacillus cereus* growth. Limiting the amount of time products are in the food safety "danger zone" (40°F -140°F) is essential as germination and outgrowth of spores can occur at temperatures from 41-122°F (Kramer & Gilbert, 1989). The effects of temperature abuse can lead to possible toxin formation.

There are four known toxins formed by *B.* cereus that can cause food poisoning. The emetic toxin (cereulide) is associated with the intoxication commonly called "fried rice syndrome". Cereulide is pH and heat stable, even at highly alkaline pH, showing inactivation at pH >9.25 when heated for 180 min at 100°C (Granum & Lund, 1997; Rajkovic, et al., 2008). Symptoms associated with the emetic toxin occur approximately 1-6 h after ingestion and generally last 6-24 h. The toxin is most often found in starchy foods with a majority of the reported cases associated with consumption of cooked rice from restaurants (Kramer & Gilbert, 1989). Restaurants are at a higher risk because large quantities of foods are made and potentially held for

long periods of time. The safety of food is in jeopardy when it is held at an improper temperature for too long.

B. cereus also produces three enterotoxins which are heat labile and produced post-infection in the small intestine (Granum & Lund, 1997). While the toxins are produced after colonization of bacteria in the lower GI tract, the symptoms are associated with people who have consumed cooked meat and vegetables. These symptoms generally appear 8-16 h after consuming the contaminated food and last 12-24 h.

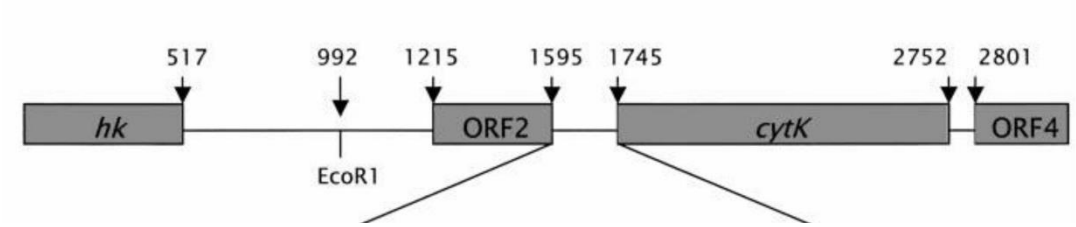

Figure 2 CytK operon. The region from 1745-2752 encodes the CytK protein *(Lund, De Buyser, & Granum, 2000)*.

One of the enterotoxins is a single component protein (CtyK, Figure 2). CytK has been shown to be highly toxic. It has been isolated from a strain of *B. cereus* that caused the death of three people (Granum & Lund, 1997; Lund, De Buyser, & Granum, 2000).

The other two enterotoxins are both three-component proteins. One of them is hemolytic (HBL, Figure 3) and the other is non-hemolytic (NHE, Figure 4). Both of the toxins need all three of the components to achieve maximum cytotoxic activity (Granum & Lund, 1997). The HBL toxin has been considered as the primary virulence factor in *B. cereus* diarrheal food poisoning because of its ability to lyse blood cells (Beecher, Schoeni, & Wong, 1995). NHE does not contain the B or L_2 components found in HBL needed for hemolytic activity, though the toxin contained a similar

component, L_1. The three components of NHE caused the same cytotoxic symptoms that HBL did (Lund & Granum, 1996).

Both types of toxin-mediated food poisoning have a short duration. Because of this, it is assumed that there is significant underreporting or misclassification as another foodborne illness. *B. cereus* causes an estimated mean 63,400 cases of foodborne illness in the US alone. The mean is estimated from the confirmed cases while factoring in the possible underreported and underdiagnosed cases (Scallan, et al., 2011).

Figure 3. HBL operon. Three component protein L_2, L_1, and B encoded by *hblC, hblD,* and *hblA,* respectively *(Granum & Lund, 1997)*.

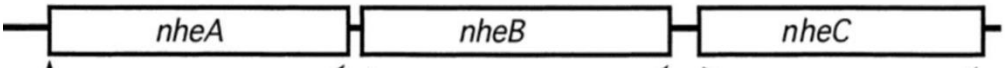

Figure 4. NHE operon. Three component protein encoded by *nheA-C (Granum, O'sullivan, & Lund, 1999)*.

Tempeh Historically

Tempeh made from soybeans has been consumed for many years in Indonesia and has gained popularity in the United States among vegetarians and vegans. Its popularity has increased because of its high protein content which is a macronutrient often lacking in plant-based diets.

Traditional production of soybean tempeh (Figure 5A) follows these basic steps: 1) removing skins (hulls) from whole beans, 2) soaking the beans to allow Lactic Acid Bacteria (LAB) proliferation to decrease the pH, 3) boiling to reduce/eliminate background microorganisms, 4) inoculation of the soybeans with a

Rhizopus spp. mold starter culture, 5) incubation until a solid, sliceable mass is formed (Steinkraus, Hwa, Van Buren, Provvidenti, & Hand, 1960; Nout, Beernink, & Bonants-van Laarhoven, 1987).

There are many variations to this basic process including an additional boiling step (Figure 5B) or soaking step (Figure 5C). The variations seem to result from the difference in location, resources, and capacity of the production facility. Even with these variations, the basic steps play a large role in tempeh completion and they have been used for many years with great success.

Current practices

Tempeh production in the US is different than the traditional Indonesian protocol. Much of the difference comes from the variation in climate and available grains. Indonesia has a humid, warm, tropical climate, and soybeans are not typically grown in Indonesia, but rather, tempeh is made from coconut meat. Additionally, most of the tempeh produced in Indonesia is fermented open to the air. These conditions help in the quick growth of mold and better control of competing microorganisms.

Though the US has a varying range of climates, replicating the conditions found in Indonesia has been a challenge. The environment plays a large role in the success of the fermentation, so the process needed to be adapted. Depending on the capability of the tempeh facility, modifications have been made in order to have a completely fermented cake. These changes include things like incubating the mold-inoculated soybeans in a more oxygen permeable container and incubating at 38°C and 75-85% RH for 18 h (Hachmeister & Fung, 1993). As with the traditional process, deviation from the appropriate processing steps can lead to foodborne illnesses.

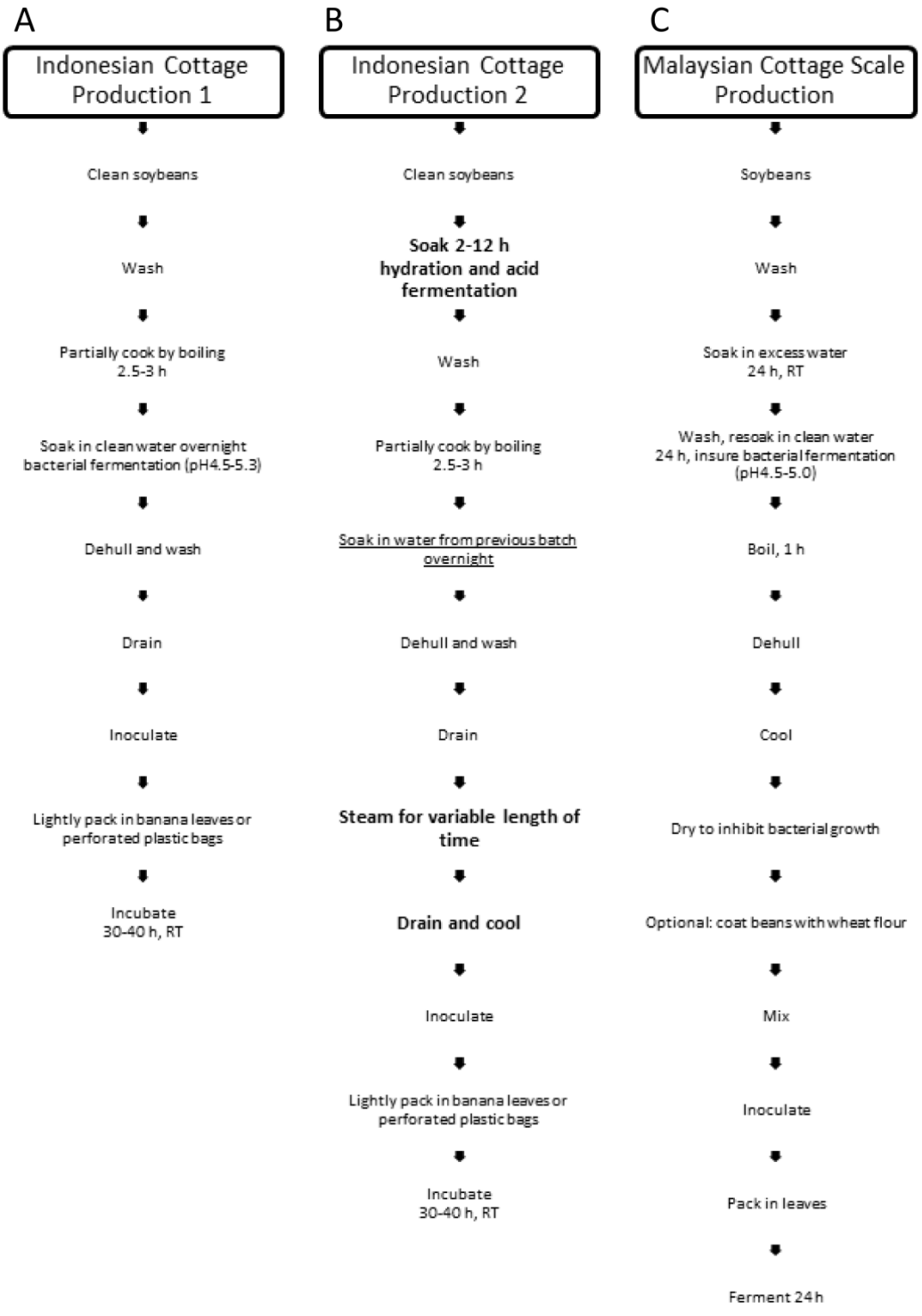

Figure 5. Tempeh production flow charts *(Steinkraus, 1996)*. Variations: Bold-faced, additional steps; Underlined, replacement for other steps.

One of the control points in safely producing tempeh is acidification of the water in which the beans will either be soaked. This can be achieved in different ways. One of the methods involves adding LAB to the soaking water and allowing sufficient time for the bacteria to grow and lower the pH (Nout, Beernink, & Bonants-van Laarhoven, 1987). The bacteria ferment the sugars found in the beans and produce lactic acid. Another way to ensure the pH is low enough is to directly acidify the water (Steinkraus, Handbook of Indigenous Fermented Foods, 1996).

Controlling pathogen growth

There are several ways to control pathogen growth in a food product. Most food products utilize several different parameters in order to achieve the best control. This technique of combined prevention methods is called hurdle technology. Hurdle technology has been used for several years, but has just recently (within the past 30 years) been studied in depth (Leistner L. , 2000).

Thermal processing itself is a reliable method of preservation, but in most products, can be the cause of off flavors. This is part of the reason why hurdle technology is preferred by food processors. With the addition of different hurdles such as pH control or water activity, the thermal treatment may be reduced (Leistner L. , 1994). Most processes use the combination of hurdles in order to achieve the required log reduction needed to control the growth of the pertinent pathogenic microorganism while maintaining quality. However, there are caveats to using hurdle technology. When using hurdle technology it is assumed that the control measures being taken are appropriate for that product.

Controls during tempeh manufacture

There are no common practices for tempeh manufacture in the US. The safety of tempeh, as with most food products, relies on proper good manufacturing practices (GMPs) and an appropriate set of standard sanitizing and operating procedures (SSOPs). Without SSOPs and GMPs in place, the risk of causing a foodborne illness increases. Many of the tempeh production steps need specific minimum operating parameters in order to make a consistently safe product.

Reducing the pH before inoculation is crucial to inhibit pathogen or spoilage growth during the *Rhizopus* fermentation step. In addition to direct inhibition of unwanted growth, the lower pH will increase the chances of quick mold growth because of the molds ability to grow at the lower pH. This can help reduce the possibility of pathogen growth by decreasing the incubation time of the mold. Many human pathogens show optimal growth around body temperature (98.6°F). Since tempeh is incubated at approximately 95°F, there is a risk of rapid pathogen growth.

Boiling the soybeans further enhances the action of the acid by reducing the bacterial load, which increases the chance of a successful fermentation. It may be fairly obvious, but if the soybeans are not inoculated with sufficient levels of *Rhizopus*, then the fermentation will fail. Inoculated soybeans are incubated until the soybean cotyledons have been penetrated by the *Rhizopus* mycelia.

Most commercial tempeh is pasteurized before it is sold, but for small-scale producers and people who might make tempeh at home, boiling may be the only heat step. This means that everything after boiling (cooling, inoculation, incubation) is at risk of post-process contamination.

The soaking step used in the traditional method of preparing the soybeans selects for acid tolerant bacteria. The spores could potentially germinate and adapt to the acid. This acid adaptation may increase the thermal tolerance of pathogenic

bacteria (Usaga, Worobo, & Padilla-Zakour, 2014). The soaking step is also time consuming. In large-scale tempeh manufacturing, the soaking step is removed. In order to achieve full hydration without soaking, the boiling time is increased to 60 min.

In a study by Nout, Beernink, and Bonants-van Laarhoven (1987) it was observed that spontaneous fermentation by LAB does not consistently occur during the soaking process, which then resulted in higher pH values (5.5-6.6). These pHs may allow the natural microbiota to outcompete the fermenting mold. Steinkraus *et al.* noted that acidification to pH 5.0 during the soaking step could prevent the undesirable growth of spoilage organisms. However, *B. cereus* and *Listeria monocytogenes* have been shown to grow at pH 5.0 and pH > 4.39, respectively, and survival of *Escherichia coli* O157:H7 and *Salmonella* spp. was observed at pH 4.4 and pH < 4.0, respectively (Kramer & Gilbert, 1989; Ingham, Buege, Dropp, & Losinski, 2004; Leyer, Wang, & Johnson, 1995; Podolak, Enache, Stone, Black, & Elliott, 2010). Therefore, additional acidification beyond pH 5.0 is recommended to prevent the growth and survival of foodborne pathogens.

Bongkrekic intoxication is an example of a foodborne illness acquired from consumption of tempeh contaminated with *Burkholderia gladioli* var. *cocovenenans*. This particular tempeh is made from coconut presscakes or coconut milk residues inoculated with *R. oligosporus*. *B. gladioli* produces bongkrekic acid, a toxin implicated for the illnesses and in some cases deaths (Garcia, Hotchkiss, & Steinkraus, 1999; Moebius, et al., 2012). Much like the recent outbreak of *Salmonella* in tempeh, the contamination occurs when the *R. oligosporus* starter culture is contaminated with the pathogenic bacteria (Moebius, et al., 2012). For this reason it is necessary to have controls to inhibit growth of pathogenic microorganisms that may be introduced post-

heat treatment as well as ensuring the tempeh starter is not contaminated with pathogenic bacteria.

It has been previously suggested (Nout, de Dreu, Zuurbier, & Bonants-van-Laarhoven, 1987; Nout, Beernink, & Bonants-van Laarhoven, 1987) that a portion of soak water from a previous production can be used as an inoculum for LAB. This procedure is called backslopping and, while it may result in a decrease in pH of the soybeans, it is discouraged as it may introduce spoilage or pathogenic organisms from a previous batch.

The current study aimed to determine the parameters needed to ensure a consistently safe product. This was done through use of two organic acids, lactic and acetic. The acids were added to the water the soybeans were boiled in to lower the pH of the soybeans. Growth of *Bacillus cereus* during tempeh incubation was utilized to establish a baseline to be used in challenge studies with the outbreak strain of *Salmonella* spp.. Because *B. cereus* is found naturally on soybeans, it is important that its growth be inhibited. In addition, because *Salmonella* has been implicated in a large outbreak of tempeh, the microbial hurdles used to enhance the safety of food should also control for this pathogen.

CHAPTER 2

MATERIALS AND METHODS

Isolation of **Bacillus cereus** *from soybeans*

Dehulled, cracked soybeans (100 g) were soaked in tap water at a ratio of 10:1 (water:soybeans). Soybeans were boiled for 5 min in a pressurized steamer and then cooled to ~50°C (liquid temperature). A solution of polymyxin B sulfate was added for a final concentration of 10 mg/L to inhibit growth of Gram negative organisms. The soybeans were incubated at 30°C for 24 h.

After incubation, the soaking liquid was plated onto Bacillus Cereus Agar (BCA) to select for *B. cereus*. The medium was supplemented with 100 units/mL polymyxin B, 25 mL/500 mL sterile egg yolk, and 20 µg/mL cycloheximide. Polymyxin B sulfate was added to inhibit Gram negative bacteria; egg yolk was added for differentiation between *Bacillus* species; cycloheximide was added to inhibit growth of mold. Plates were incubated at 30°C for 18 h. Colonies were selected and re-streaked for isolation onto Plate Count Agar (PCA), then incubated at 30°C for 36 h. Single colonies were inoculated into 5 mL TSB (Tryptone Soy Broth) tubes and incubated for 18 h at 35°C, 225 rpm. Overnight culture (850 µl) was mixed with sterile glycerol (150 µl) and kept as freezer stocks at -80°C.

DNA Extraction from Soybean Isolate

DNA extraction was performed using the Ultraclean Microbial DNA Isolation kit (MOBIO Laboratories, Carlsbad, CA) following the kit protocol. The DNA was used for 16S PCR amplification with lab primers (forward: 5' – AGAGTTTGATCCTGGCTCAG; reverse: 5' – AAGGAGGTGATCCAGCCGA). The conditions were as follows: 1 cycle at 94° for 3 minutes; 30 cycles of 94° for 1 minute, 37° for 2 minutes, 72° for 1.5 minutes; 1 cycle at 72° for 8 minutes. Once the

PCR cycle was finished, the PCR products were run on a 0.7% agarose gel with .005% ethidium bromide. The resulting bands were excised and extracted with the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). Purified fragments were sent to Cornell University Life Sciences Core Laboratories Center to be sequenced. PCR amplification of *gyrB* was also performed as previously shown (Lee, Churey, & Worobo, 2009).

Mold and Bacterial Cultures used in Challenge Studies

Five strains of *B. cereus* (Table 1), including the strain isolated from soybeans, were used in food challenge studies. The bacteria were plated from freezer stocks onto TSA incubated at 30°C for 18-20 h. Plates were kept for no more than 14 days before re-streaking. When needed, single colonies were taken using a sterile loop and inoculated in TSB (5 ml). The tubes were incubated for 18 h at 35°C, 225 rpm.

Rhizopus oligosporus and *Rhizopus oryzae* were cultured on PDA (Potato Dextrose Agar) adjusted to pH 3.5 with tartaric acid. Both were incubated at 35°C until they reached sporulation. Sporulation varied between the two with *R. oligosporus* taking 72-96 h and *R. oryzae* taking 120-168 h. Mold plates were not used more than 14 days after initial incubation. Sterile swabs were used for subculturing the molds. For use as tempeh starter culture, plugs were taken with sterile 1 mL pipette tips with a 7mm diameter.

Table 1. *B. cereus* strains used. All strains (except Bean isolate) were previously isolated and plated from lab freezer stocks.

11778
P2E018
F4810
F4552
Bean (isolated for this study)

Soybean processing

Soybeans (1 kg) were boiled in water (9 kg) that was 1) unacidified, 2) acidified with lactic acid, or 3) acidified with acetic acid. Equilibrated pH was taken prior to inoculation with bacteria and mold for all measurements. Equilibrated pH is the pH of the entire bean and was determined by grinding 80.0 g of cooked and cooled soybeans with 8.0 g water. Lactic acid was used to generate water with the equilibrated pH of 4.8, 4.6, or 4.4. The acetic acid was added in amounts to give a finished, equilibrated pH of 5.9, 5.7, or 5.5. Beans were boiled in an open-air steam jacketed kettle. Once boiling, the soybeans were cooked for an additional 60 min. After 60 minutes, the water was decanted and the beans were poured into a large pan and allowed to cool to room temperature in a biosafety cabinet.

Inoculation of soybeans with mold and bacterial cultures

Cooled soybeans (150.0 g) were measured and placed into sterile, plastic boxes covered in foil. For each pH there were 10 total boxes. One was designated as the uninoculated control, three were inoculated with *B. cereus* only, three were inoculated with *R. oligosporus* and *B. cereus*, and three were inoculated with *R. oryzae* and *B. cereus*. The inoculation was assigned using the randomize function in Microsoft Excel.

For the boxes inoculated with mold, the previously mentioned plugs were mixed into the beans first and then the soybeans were inoculated with *B. cereus* (150 µl) and mixed again to increase dispersion of the mold. Tempeh was incubated with a loose foil covering at 35°C.

Survival of **B. cereus** *in Acidified Tempeh*

Soybeans (10 g) and 90 g of sterile peptone water were weighed into sterile sampling bags and stomached for 2 min at 230 rpm. The resulting solution was spread plated at appropriate dilutions on BCA.

Survivors were sampled at 0 h, 12 h, 24 h, 48 h, and 72 h or until fermentation was completed. All boxes without mold inoculation were sampled each time until all fermentations were completed or failed. Fermentation was deemed acceptable by visual inspection. The soybeans must have been a solid mass, covered and penetrated by the mycelia.

Enumeration of **B. cereus**

Counts are presented as $\log_{10}$ of CFU/g beans. Colonies were counted if they had characteristic morphology for the *Bacillus cereus* media. The putative *B. cereus* colonies were identified by their ability to lyse the egg yolk, creating a precipitate that showed as a milky halo. There also needed to be an indication of pH change due to fermentation of mannitol.

Data Analysis

JMP Pro 10 (SAS, Cary, NC) was used to run statistical analysis. Mixed effects modeling was used as the test for endpoint analysis of *B. cereus* growth.

CHAPTER 3

RESULTS AND DISCUSSION

Optimization of protocols

The pH modification of the soybeans to the experimental pH values was challenging to control, due to the high protein content and slow equilibration of the soybeans over time. In order to keep the process consistent, it was necessary to test varying amounts of acetic and lactic acid. Acidification was checked after boiling the soybeans by the same procedure as mentioned above. Target pHs were based on the study by Nout, Beernink, & Bonants-van Laarhoven (1987) titled "Growth of *Bacillus cereus* in soyabean tempeh". Furthermore, acidification was less consistent when soaking the soybeans in acidified water prior to cooking. Acidification consistency was improved after adopting the large-scale production process of increased boiling time, without a pre-soak.

The viscosity of lactic acid caused problems with volumetric measurement of acid. Because of this, the lactic acid was weighed and mixed with a portion of the water used to cook the soybeans before pouring in to the remaining water. This was not necessary for the acetic acid, as it can be measured accurately with a pipette.

In order to achieve complete fermentation by *Rhizopus oryzae* in any of the treatments, the inoculum had to be doubled. When trying to create tempeh from one sporulated mold plug (7 mm^2), the *R. oryzae* did not produce tempeh that was acceptable (solid, sliceable mass). Increasing the inoculum to 14 mm^2 resulted in a majority of the replicates being acceptable.

The amount of cycloheximide used as a mold inhibitor in plating for enumeration of *B. cereus* also needed to be optimized. A filter sterilized stock solution of cycloheximide was added in increasing concentration to sterile *B. cereus* agar at

55°C. The final concentration (20 μg/ mL) was chosen as the lowest concentration at which *B. cereus* grew, but *R. oligosporus* was inhibited.

Effect of treatments on growth of* Rhizopus *spp.

In order for the treatments to be effective, the addition of acid should not prevent the mold from completing fermentation. Acidification of soybeans prior to inoculation with mold was used as to inhibit the growth of *Bacillus cereus.* Inoculation with *R. oligosporus* or *R. oryzae* as starter culture produced tempeh of varying quality depending on the treatment (Table 2). Quality was characterized by completion of *Rhizopus* spp. fermentation. If there were portions of the soybeans that had no visible mycelial growth while the rest of the beans were covered in sporulated *Rhizopus* spp. the fermentation was deemed incomplete.

For all pH levels of lactic acid tested, there was visible growth of *R. oligosporus* at the 12 h time point. Visible growth of *R. oryzae* in this case was not seen until 24 h after initial incubation, indicating increased time needed for complete fermentation. For both the acetic and lactic treatments, acidification did not change the amount of time for completion of fermentation by *R. oligosporus.* However, when the soybeans were acidified with acetic acid, it took longer than 12 h for there to be any visible growth of *R. oligosporus*, suggesting a slightly slower growth pattern. In the case of the acetic acid treatment at pH 5.9, *R. oryzae* growth was visible at 24 h but at 48 h any visible mold was sporulated. In the unacidified treatment, neither mold completed fermentation. At 24 h, visible mold was sporulating, but there were also sections of soybeans that did not show any visible mold growth.

Table 2. Observed completion of tempeh fermentation by *R. oligosporus* and *R. oryzae*. '+': completed, '-': incomplete. Fermentation completed in 24 h for *R. oligosporus* lactic and acetic acid treatments. Fermentation completed in 48 h for *R. oryzae* lactic treatment and at pH 5.5 and pH 5.7 acetic acid treatment.

Treatment	pH	*R. oligosporus*	*R. oryzae*
Unacidified	6.7*	-	-
	6.7**	-	-
Lactic	4.8	+	+
	4.6	+	+
	4.4	+	+
Acetic	5.9	+	-
	5.7	+	+
	5.5	+	+

* Soybeans sterilized prior to inoculation
**Soybeans inoculated without sterilization

Effect of treatment on growth of B. cereus

The lactic acid had an overall inhibitory effect on *B cereus* (Figure 6). The log counts of *B. cereus* were kept below two log CFU g^{-1}. Adding lactic acid lowered the pH which controls the growth of some microorganisms, while selecting for Lactic Acid Bacteria (LAB), which produce a variety of bacteriocins. Bacteriocins inhibit the growth of closely related bacteria so it is possible that low pH is not the only factor affecting the growth of *B. cereus* (Klaenhammer, 1993). The effect of acetic acid on *B. cereus* was not sufficient to inhibit the bacteria from growing (Figure 7). The bacteria grew three to five logs above the initial inoculum.

For both the unacidified and acetic acid treatments, the texture of the visibly unfermented soybeans was different when compared to the uninoculated control. The soybeans were a much darker, yellow-brown color with a shiny, white, mucous-like coating that was sticky to the touch, while the soybeans in the uninoculated controls were loose and still light yellow. This change in texture suggests the possibility that the beans were being fermented by *B. cereus.*

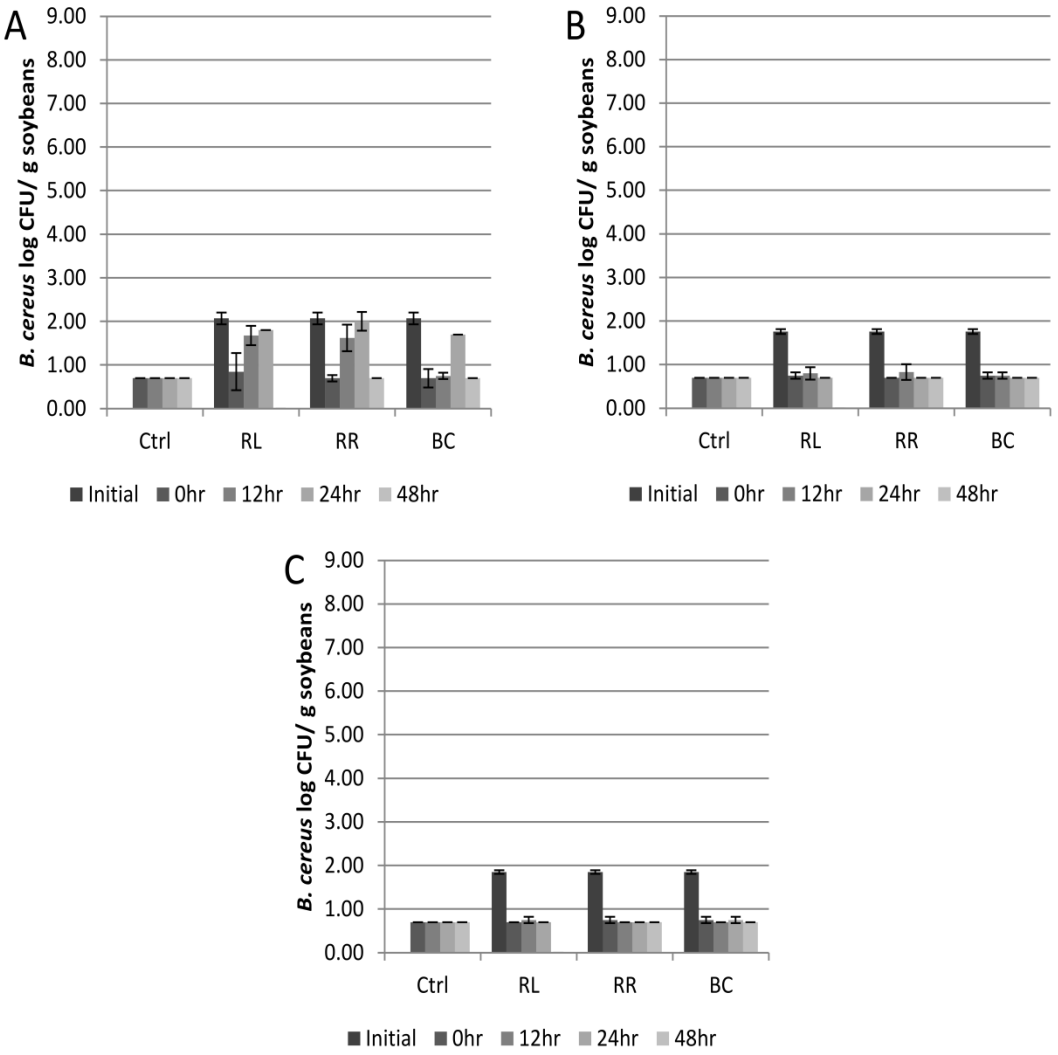

Figure 6. Graphs of recovered *B. cereus* cells for lactic acid trials. pH 4.8, 4.6 and 4.4 (A, B, and C, respectively). Error bars represent standard deviation (n = 3). No value reported for RL at 48 hr because complete fermentation occurred at 24hr. Values below 1 log represent plate counts with an average <1 cell at 10^{-0} dilution. Ctrl = Uninoculated control, RL= *Rhizopus oligosporus* + *B. cereus,* RR = *Rhizopus oryzae* + *B. cereus,* BC = *B. cereus* alone.

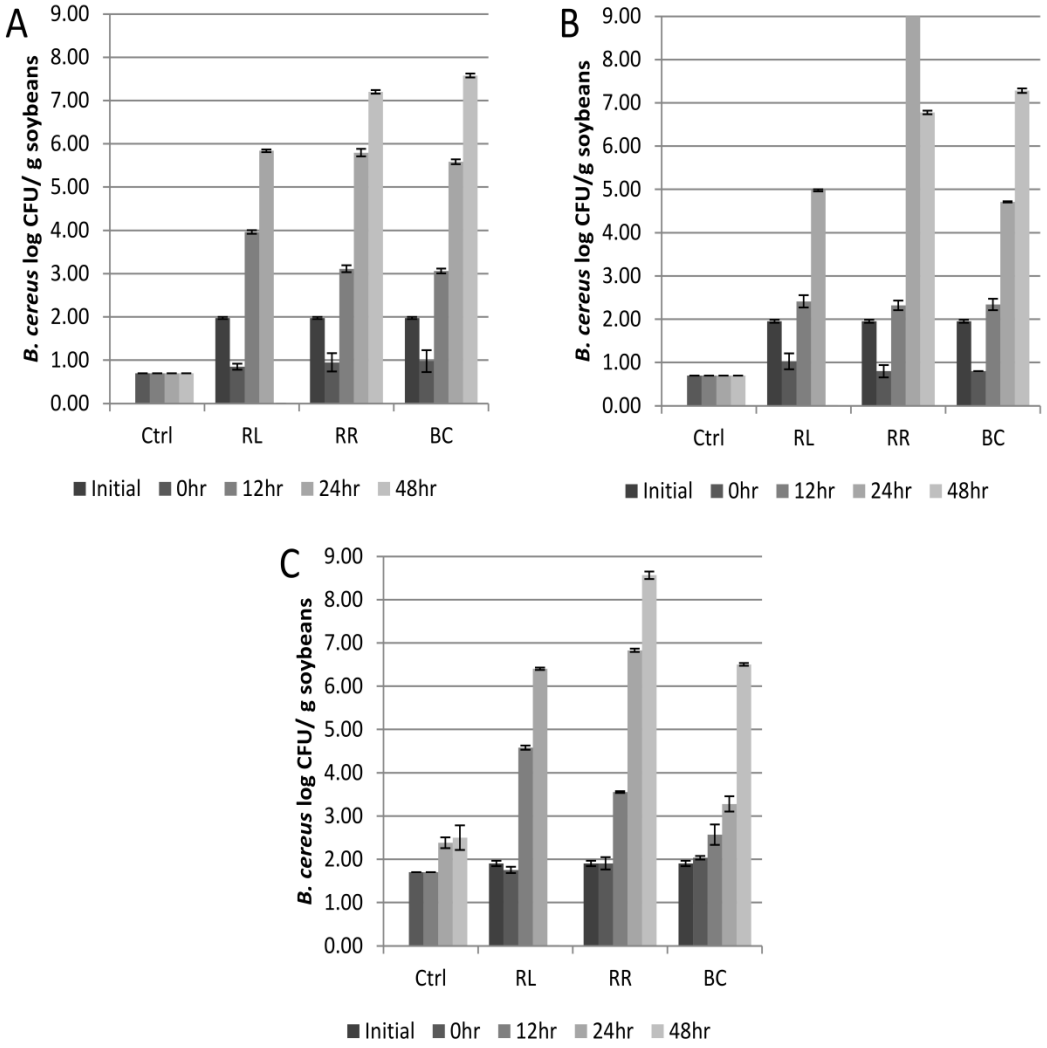

Figure 7. Graphs of recovered *B. cereus* cells for acetic acid trials. pH 5.9, 5,7 and 5.5 (A, B, and C, respectively). Error bars represent standard deviation (n = 3). No value reported for RL at 48 hr because complete fermentation occurred at 24hr. Values below 1 log represent plate counts with an average <1 cell at 10^{-0} dilution.Ctrl = Uninoculated control, RL= *Rhizopus oligosporus* + *B. cereus,* RR = *Rhizopus oryzae* + *B. cereus,* BC = *B. cereus* alone.

Fermentation of soybeans by *Bacillus subtilis* in natto production is characterized by a similar sticky texture (Wei, Wolf-Hall, & Chang, 2001). A comparison of endpoint *B. cereus* population shows the trends between the treatments (Figure 8). *B. cereus* was below detectable limit when acidification occurred with lactic acid. Acidification with acetic acid had little effect on inhibition of *B. cereus* growth when compared to the unacidified control, and in some cases exceeded the growth recorded for the unacidified treatment. There was less than a 5 log reduction of *B. cereus* for most of the acetic acid treatment when comparing the end population to the initial inoculum.

There was a statistically significant difference in the effect of the treatments on *B. cereus* growth. Counts for the lactic acid treatment and the control replicates were, on average, lower than one log. These data were removed from the statistical analysis, as they are significantly different from all values. The differences between the unacidified and acetic acid treatments are shown below in Table 3. The log counts presented are those of the endpoint *B. cereus* population for those replicates. However, it should be noted again that neither of the *Rhizopus* spp. completed fermentation in the unacidified treatments and *R. oryzae* did not complete fermentation for the acetic acid treatment at pH 5.9.

The interaction between treatment (unacidified, acetic acid, or lactic acid) and inoculum (*R. oligosporus* + *B. cereus, R. oryzae* + *B. cereus, or B. cereus* alone) showed there was a significant difference between the effects of acetic acid for the three inocula ($P < 0.05$). This means the log count of *B. cereus* depended on which *Rhizopus* species was used. The effect of the acid was significantly greater (less growth) when used in combination with *R. oligosporus.*

Figure 8. Endpoint comparison of *B. cereus* population per treatment. Unacidified treatment based on counts after 24 h though fermentation was not complete. Error bars represent standard deviation (n = 3). S = Sterilized soybeans prior to inoculation, NS = Not sterilized prior to inoculation. Ctrl = Uninoculated control, RL= *Rhizopus oligosporus* + *B. cereus,* RR = *Rhizopus oryzae* + *B. cereus,* BC = *B. cereus* alone.

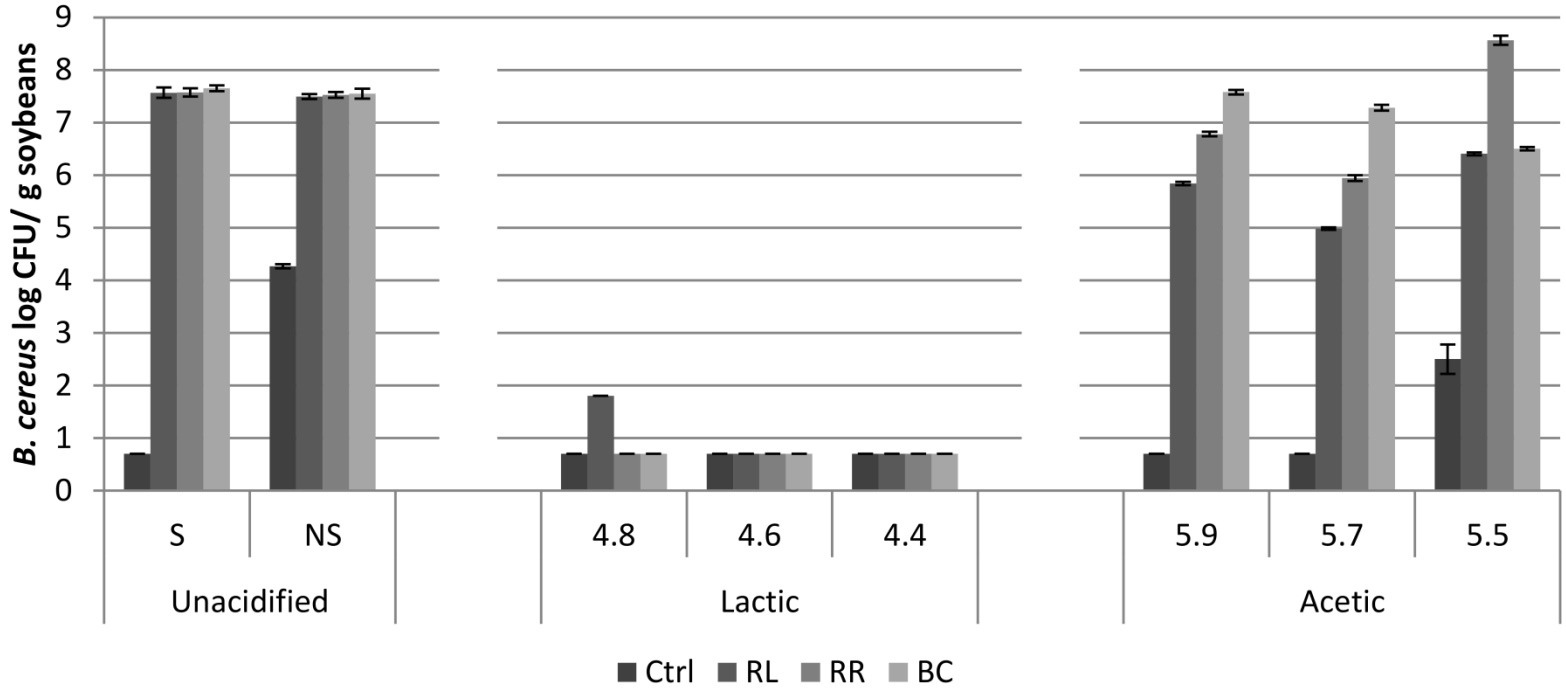
B. cereus log CFU/ g soybeans
9
8
7
6
5
4
3
2
1
0
S
NS
4.8
4.6
4.4
5.9
5.7
5.5
Unacidified
Lactic
Acetic
Ctrl
RL
RR
BC

Table 3. *B. cereus* endpoint population with different treatments. Values not sharing a common lowercase letter represent significantly different values ($P<0.05$) based on post hoc multiple comparisons with a Tukey correction following mixed effects modeling.

		Inoculum (*B. cereus* log CFU/ g soybean ± stdv)		
Treatment	pH	*R. oligosporus* + *B. cereus*	*R. oryzae* + *B. cereus*	*B. cereus*
Unacidified	6.7*	7.57 ± 0.097^{b}	7.57 ± 0.076^{b}	7.65 ± 0.054^{b}
	6.7**	7.49 ± 0.050^{b}	7.53 ± 0.053^{b}	7.55 ± 0.096^{b}
Acetic	5.9	5.84 ± 0.033^{d}	6.78 ± 0.041^{a}	7.58 ± 0.045^{d}
	5.7	4.98 ± 0.023^{f}	5.94 ± 0.056^{c,d}	7.28 ± 0.053^{b,c}
	5.5	6.40 ± 0.031^{e}	8.56 ± 0.087^{b,c}	6.50 ± 0.030^{b}

Though the differences between the values are considered statistically significant, it should be said that most of the log reductions would still not be considered sufficient for a safe food product. When looking at the acetic acid and unacidified treatments, the difference between the two treatments is significant, but the overall log reduction was still too low for each. The effect of lactic acid on the inocula was not significant, but the log counts were below 10^2 CFU/g, which is greater than a 5 log reduction. It has been estimated that the infective dose is approximately 10^5- 10^8 cells for the emetic syndrome and 10^5- 10^7 cells for the diarrheal syndrome (Granum & Lund, 1997). Because of the quick doubling time of *B. cereus* it is necessary to inhibit outgrowth of endospores and replication of vegetative cells.

CHAPTER 4

CONCLUSIONS

Several factors made the inhibition of *B. cereus* a challenge. First, soybeans can vary in protein and starch content. Because of this variation, it can be difficult to consistently control the pH. Consistent acidification was achieved by changing the process of tempeh manufacture. Soaking the beans is typically employed in traditional tempeh production methods for the growth of Lactic Acid Bacteria (LAB), which lower the pH of the soybeans due to the production of lactic acid by LAB during the soak step. This natural acidification proved to be inconsistent and time consuming when commercially producing tempeh in the United States. In order to achieve a consistent pH, the soaking step was removed and the beans were boiled in acidified water for 60 min, to allow for adequate acidification as well as hydrating the soybeans for tempeh production.

Second, acidification of soybeans during cooking is essential to prevent growth of *B. cereus* during the mold fermentation step. The boiling process to hydrate the soybeans is insufficient to inactivate spore forming bacteria such as *B. cereus*, and fermentation incubation temperatures would allow for the proliferation of the germinated *B. cereus*. Acidification of the beans serves as a hurdle to prevent the growth of *B. cereus*, but allow for *Rhizopus* spp. to carry out the tempeh fermentation completely. This acidification should be done by adding lactic acid to a final equilibrated pH of at least 4.8. There is a difference in log counts of approximately 10^7 units between acidified and unacidified tempeh in the finished tempeh. When acidifying to pH 4.6 or 4.4, the results were similar. However, using excess acid would result in additional expense to the tempeh manufacturing, so reaching the minimum level is cost effective for the tempeh industry.

Third, the use of acetic acid will not completely inhibit *B. cereus* from growing. The lowest pH of acetic acid used in this study was above the level used for food safety (pH 4.8), however, using acetic acid can also prevent the *Rhizopus* spp. from fermenting the soybeans to an acceptable product.

If pasteurization is not utilized, the last kill step is prior to inoculation with *Rhizopus* spp. The chances of post-processing contamination are amplified and the risk of foodborne illness increases. As part of SSOPs, steps should be in place to ensure the starter culture does not become contaminated with pathogens. This will reduce possible contamination of the tempeh once the soybeans are incubating.

CHAPTER 5

FUTURE WORK

Bacillus cereus *challenge study*

The effect of acid (lactic or acetic) was not completely clear in the present study. Though there was a definite effect of the lactic acid on the growth of *B. cereus,* it is not clear if it was the acid or the pH that was inhibiting growth. In order to compare the acids, the pH needs to be the same for both treatments. As stated above, the pH levels in this study were chosen based on a previous study. The pH values for the acetic acid treatment should be lowered to that of the lactic acid treatment to see effectiveness. Additionally, the pH for lactic acid should be increased to determine the maximum pH at which inhibition occurs.

Salmonella *challenge study*

The point of this study was to find the highest pH level (least acidic) at which tempeh could be acidified and *B. cereus* was inhibited. This pH should be the starting point for challenge studies with the outbreak strain of *Salmonella* from the North Carolina tempeh outbreak. Determining the amount of acid that inhibits growth of the *Salmonella* while producing acceptable tempeh is another experiment that would lead to critical parameters for the safe production of commercial tempeh. This study could also be extended to include other major pathogens such as *E. coli* O157:H7 and *Listeria monocytogenes.*

***Optimizing growth of* Rhizopus oryzae**

In addition to looking at the effects of pH on pathogen survival, the growth of *R. oryzae* should be studied to find optimal growth conditions in acidified soybeans. When incubating at 35°C, *R. oryzae* completed fermentation no sooner than 48 h.

While *R. oligosporus* grows well at 35°C, *R. oryzae* may grow better at a lower or higher temperature.

Tempeh production with other grains

This study focused on tempeh made from soybeans though tempeh can be made utilizing several other grains. The effect of the grains would need to be determined if the acidification parameters established for *B. cereus* inhibition in soybeans would be sufficient with alternative grain types used in tempeh production. It may be that other grains would require different amounts of acid depending on the protein content.

Tempeh acceptability

Other important experiments are sensory tests to gauge acceptability of tempeh produced with the two different molds. Due to the slower fermentation of *R. oryzae,* background microflora, specifically LAB, could grow. This suggests that the perceived taste difference is caused by background growth and not an effect of the mold growth. If there is not a difference between the tastes then it should be suggested to use *R. oligosporus* due to the quicker incubation time. This would not only be the safer choice in terms of risk of illness, but also saves time and frees up space that could be used for the next batch of tempeh. In addition to sensory tests for the two *Rhizopus* spp. a test comparing the sensory effects of commercial pasteurization could be done. Again, if there is not a difference pasteurization of the fully formed tempeh should be recommended.

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