Summer 8-2021

Assessing the Efficacy of Bacteriological Analytical Manual (BAM) Enrichment Broths for Detection of *Salmonella* spp. in Meat Analogs

Georgia Sampson
Chapman University, samps112@mail.chapman.edu

Assessing the Efficacy of Bacteriological Analytical Manual (BAM) Enrichment Broths for Detection of *Salmonella* spp. in Meat Analogs

A Thesis by

Georgia Logan Sampson

Chapman University

Orange, CA

Schmid College of Science and Technology

Submitted in partial fulfillment of the requirements for the degree of

Master of Science in Food Science

August 2021

Committee in charge:

Rosalee Hellberg, Ph.D., Chair

Anuradha Prakash, Ph.D.

Lilian Were, Ph.D.

The thesis of Georgia Logan Sampson is approved.

Rosalee S. Hellberg

Rosalee Hellberg, Ph.D., Chair

Anuradha Prakash

Anuradha Prakash, Ph.D.

Lilian Were

Digitally signed by Lilian Were
Date: 2021.06.21 17:49:15 -07'00'

Lilian Were, Ph.D.

June 2021

Assessing the Efficacy of Bacteriological Analytical Manual (BAM) Enrichment Broths for Detection of *Salmonella* spp. in Meat Analogs

by Georgia Logan Sampson

ACKNOWLEDGMENTS

I would like to thank my thesis committee members, Dr. Hellberg, Dr. Were, and Dr. Prakash, for guiding me through my thesis. I would also like to thank our FDA collaborators, Dr. Williams-Hill, Shannon Ruelle, and Lieuchi Phan for contributing their expertise and facilities to the project. I would also like to thank Ashley Whelpley for her assistance, Dr. Toto Pacioles for guidance in statistical analysis, and Grace Marquis for assisting me in the lab.

ABSTRACT

Assessing the Efficacy of Bacteriological Analytical Manual (BAM) Enrichment Broths for Detection of *Salmonella* spp. in Meat Analogs

by Georgia Logan Sampson

Plant-based meat alternatives are gaining popularity as the number of market options increases. However, the recent appearance of these products on the market post-dates the validation of the current approach for isolating *Salmonella* from meat analogs. Therefore, the objective of this study was to compare the efficacy of the currently used Bacteriological Analytical Manual (BAM) pre-enrichment broth (lactose broth) with two additional broths (universal pre-enrichment broth and buffered peptone water) for the detection of *Salmonella* in five plant-based burger products. Each burger product was spiked with a *Salmonella* serotype (*S.* Enteritidis or *S.* Agona) that has previously been linked to one or more of the protein sources in the product. The U.S. Food and Drug Administration (FDA) Matrix Extension Verification Procedure section 5.1.1 was followed for the verification of each broth. Seven replicates of each of the five selected plant-protein burgers were spot-inoculated with 30 or less colony-forming units (CFU) of *Salmonella enterica* per 25 g of product and enriched in each broth. An additional seven non-inoculated replicates were tested with all broths. Pathogen recovery was evaluated using the methods described in BAM and *Salmonella* was confirmed with real-time PCR, using two DNA templates per sample. Comparison of the number of positive real-time PCR detections for *Salmonella* across all three broths and five brands indicated no significant difference (p-value > 0.05) between the effectiveness of each broth. The greatest level of detection was found with LB, in which 95.71% of DNA templates tested positive for *Salmonella*, followed by BPW and UP broth, which each had an 88.57% detection rate. When the broths were compared for

effectiveness in each product separately, LB showed a significantly greater percentage of positive detections (92.86%) in Beyond Meat™ samples compared to UP (50%). However, none of the other products showed significant differences in detection among the three broths. In conclusion, the findings demonstrate that LB, the current BAM pre-enrichment broth, is an effective pre-enrichment for the detection of *Salmonella* in meat analogs formulated with various plant-proteins.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Meaning
AD	*Salmonella* AD media
BAM	Bacteriological Analytical Manual
BHI	Brain Heart Infusion Broth
BPW	Buffered Peptone Water
BS	Bismuth Sulfite Agar
CDC	Centers for Disease Control and Prevention
ERE	Estrogen Response System Elements
FDA	Food and Drug Administration
GFI	Good Foods Institute
GMO	Genetically Modified Organism
GMP	Good Manufacturing Processes
HE	Hektoen Enteric Agar
IRI	International Research Institute
LAB	Lactic Acid Bacteria
LB	Lactose Broth
LGC	Least gelatinization concentration
LIA	Lysine Iron Agar

NB	Nutrient Broth No. 2
PCR	Polymerase Chain Reaction
PPPB	Pea-processing by-product
RV	Rapport Vassiliadis (RV) broth
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSI	Triple Sugar Iron Agar
TT	Tetrathionate Broth
UP	Universal pre-enrichment broth
VBNC	Viable but Non-Culturable
XLD	Xylose Lysine Deoxycholate agar

1. Introduction

U.S. retail sales of plant-based meat analogs increased 45% between 2019 and 2020, reaching USD $1.4 billion in 2020 (Gaan, 2021). The global meat-analog market was valued at USD $3.3 billion in 2019 and is expected to grow 19% from 2020 to 2027 (GVR, 2020). Meat analogs are plant- or fermentation-based products that simulate properties of animal meat, such as flavor, appearance, texture, and nutrition content (Pietsch et al., 2017). The increase in sales of meat analogs can largely be attributed to consumer interest in a health-conscious diet and increased awareness of the environmental sustainability of plant-based meat alternatives (Yuliarti et al., 2021). According to the International Research Institute (IRI), the top five meat analog brands in terms of sales in 2019 were, in descending order: Morningstar Farms™, Gardein™, Beyond Meat™, Boca, ™ and Quorn™ (Grzebinski, 2020).

Among the various types of meat alternative products available, meat analog burgers have experienced high consumer demand. These products utilize a variety of plant and/or fungal protein sources and are often produced using extrusion. In the last decade (2010-2020), meat analog companies have differentiated themselves in the competitive meat analog market through the use of diverse protein ingredients (Ismail et al., 2020). The Morningstar Farms™ Meat Lovers Vegan Burger and BOCA Extra Large All American Veggie Burger contain wheat gluten, soy protein isolate, and soy flour as protein sources; Gardein™ utilizes textured pea protein, textured wheat protein, and wheat gluten in their Ultimate Plant-Based Burger product; Beyond Meat uses pea protein isolate, mung bean and rice protein in the Beyond Burger®; and Quorn™ utilizes mycoprotein, egg white**,** wheat flour, and milk protein in their Meatless Gourmet Burgers. Another popular meat analog burger product is the Impossible™ Burger, produced by Impossible Foods™, which has partnered with several fast-food restaurants and was

made available for purchase in select grocery stores in September 2019 (Jiang, 2019; Nagarajan, 2020). The Impossible™ Burger differs from the aforementioned meat analog products as it combines soy and potato protein. Understanding the food matrix and microorganisms associated with major protein ingredients in meat analogs can elucidate pathogens of concern and inform practices to minimize these hazards.

Salmonella enterica is the top bacterial cause of foodborne illness in the United States, with an estimated 1.35 million infections each year (CDC, 2020). *S. enterica* is a Gram-negative, non-spore-forming facultative anaerobe that is widespread in nature. The main reservoir for *S. enterica* is the intestinal tract of warm- and cold-blooded animals (FDA, 2012). The bacterium is spread through the fecal-oral route and water containing animal feces can contaminate nearby crops (Andino & Hanning, 2015). Often, *S. enterica* is associated with poultry and egg products, however, outbreaks have also been linked to non-animal sources such as sprouts, raw flour, fruits, nuts, and vegetables. Between 2008 and 2021, *Salmonella* outbreaks were linked to numerous products containing plants or plant proteins, such as animal and poultry feed, mung bean sprouts, pet food, peas, and wheat puff cereal (Andino & Hanning, 2015; CDC, 2008, 2018, 2019; FDA, 2015a, 2021; McCallum et al., 2013; Wierup & Häggblom, 2010). Furthermore, *Salmonella* contamination has occurred in extruded products such as pet food, breakfast cereals, flour and crackers (FDA, 2018b, 2018d, 2019a, 2020c, 2020d). While outbreaks involving meat analog products have not yet been reported in the United States, the ingredients in these products are susceptible to *Salmonella* contamination. *Salmonella* is likely to be inactivated by common meat analog processing methods, such as extrusion; however, the product may still be at risk due to improper processing, processing failures, and post-processing contamination (Anderson et al.,

2017). With the increasing demand and consumption of meat analogs, *Salmonella* detection methods must be validated to ensure the safety of consumers.

According to the U.S. Food and Drug Administration (FDA)'s Bacteriological Analytical Manual (BAM), *Salmonella* can be detected and isolated in foods using pre-enrichment broth, followed by selective enrichment, selective plating, and confirmation (FDA, 2020a). The pre-enrichment broth is a non-selective medium used to resuscitate injured cells to a healthy state, allowing for proliferation and subsequent detection of these cells (Budu-Amoako et al., 1992; Jacobson et al., 2017). The BAM method for the isolation of *Salmonella* from meat analogs calls for pre-enrichment using lactose broth with added surfactants, Tergitol Anionic 7, or Triton X-100 (FDA, 2020a). However, this approach has not been validated for use with the wide variety of meat analogs available in the marketplace. Therefore, research is needed to determine the most effective pre-enrichment broth for the isolation of *Salmonella* from various plant-based meat analogs.

Salmonella is tolerant to acidic conditions and therefore can outcompete various microbes at a low pH. Food samples tested for *Salmonella* are often enriched with lactose broth (LB) because it does not contain buffers and therefore does not maintain a stable pH. This potentially favors the proliferation of *Salmonella*, as *Salmonella* can survive at a pH as low as 3-4 whereas most pathogenic organisms cannot (Jacobson et al., 2017; Keerthirathne et al., 2016). Ultimately, if the pH is decreased during enrichment with lactose broth, *Salmonella spp*. can outcompete and better survive compared to other microorganisms (Jacobson et al., 2017). However, Jacobson et al. (2017) found that pre-enrichment with a broth containing buffers such as buffered peptone water (BPW) and universal pre-enrichment (UP) broth, was more effective in isolating and detecting *Salmonella* in leafy green produce and herb samples compared to LB due to the ability

to maintain a stable pH. Leafy green vegetables may have required a more stable pH during enrichment due to higher amounts of lactose fermenting bacteria in the samples. If a greater concentration of lactose fermenting bacteria were present in the samples, the pH may have dropped below *Salmonella* tolerance (3-4 pH) killing or injuring the *Salmonella* cells, and resulting in lowered detection in the unbuffered pre-enrichment broth. The BAM recommends the use of BPW for the isolation of *Salmonella* from animal foods, which often contain plant protein sourced from soy, legumes, and/or wheat. UP broth, on the other hand, is recommended by the BAM for isolation of *Salmonella* from fresh leafy vegetables, herbs, and sprouts, as it has the highest buffering capacity in comparison to LB and BPW (FDA, 2020a; Jacobson et al., 2017). Additionally, UP broth contains added nutrient compounds such as sodium pyruvate, ferric ammonium citrate, and magnesium sulfate, all of which aid in the proliferation of pathogenic cells (Hammack et al., 2008).

The objective of this study was to compare the efficacy of three different pre-enrichment broths (i.e., LB, UP broth, and BPW) in the isolation and detection of *Salmonella* in plant-based burger products containing a variety of protein sources. It was expected that spiked meat analogs containing soy and pea protein sources with known antimicrobial activity would require a more nutrient-dense pre-enrichment broth, such as universal pre-enrichment broth, to combat the antimicrobial effects of the ingredients. Additionally, it was expected that meat analogs with soy and mung bean protein, which have previously been associated with lactose-fermenting bacteria, would perform poorly with lactose broth, as the presence of lactose-fermenting bacteria may lower the pH. Lastly, it was expected that UP broth would show the greatest versatility for *Salmonella* detection across the various meat analogs brands tested, as it contains added

tryptone, dextrose, sodium pyruvate, magnesium sulfate, and ferric ammonium citrate, and therefore is more nutrient dense compared to LB and BPW.

2. Review of Literature

2.1. Pre-enrichment broth for the detection of *Salmonella*

Pre-enrichment broth is a non-selective medium utilized as a first step in the detection and isolation of pathogens in food matrices. The purpose of pre-enrichment broth is to promote the growth of the target pathogen to detectable levels and resuscitate any injured cells. Food processing techniques, such as the use of heat, low pH, and/or chemical additives such as sodium benzoate, potassium sorbate, sodium nitrate, etc., can result in sub-lethal damage to microorganisms present in the food, causing microbial cells to become physiologically impaired or injured (Budu-Amoako et al., 1992; Chen et al., 2019; Knicky & Spörndly, 2015).

Selective enrichment, which follows pre-enrichment during the detection of pathogenic organisms, contains substances that are selective for the target organism and inhibitory towards non-target organisms. Selective enrichment enhances the detection of the targeted pathogen over other organisms potentially present in the food. However, the selective agents are sometimes toxic towards the target organism, causing the death of injured cells and/or inhibiting the repair of injured cells during the detection and isolation process (Budu-Amoako et al., 1992). Therefore, a pre-enrichment step is necessary for the rehabilitation and proliferation of any injured cells before selective enrichment (Taskila et al., 2012). The use of selective enrichment without a pre-enrichment step can cause an underestimation of the target pathogens present, leading to an overestimation of the effectiveness of processing techniques.

Food samples tested for *Salmonella* are often pre-enriched with lactose broth (LB), but pre-enrichment with a broth containing buffers, such as buffered peptone water (BPW) and universal

pre-enrichment (UP) broth, has also been successful at isolating *Salmonella* in certain foods (Jacobson et al., 2017). **Table 1** lists the ingredients for LB, UP broth, and BPW, as listed in BAM Chapter 5 for the detection of *Salmonella* in varying food matrices (FDA, 2018c).

Table 1. Formulation of pre-enrichment media listed in BAM Chapter 5 used for the detection of *Salmonella* in various food matrices.

Media	Ingredient	Amount	Final pH
Buffered peptone water	Peptone	10 g	7.2 ± 0.2.
	Sodium chloride	5 g	
	Disodium phosphate	3.5 g	
	Mono-potassium phosphate	1.5 g	
	Distilled water	1 L	
Lactose broth	Beef extract	3 g	6.9 ± 0.2
	Peptone	5 g	
	Lactose	5 g	
	Distilled water	1 L	
Universal pre-enrichment broth	Tryptone	5 g	6.3 ± 0.2
	Protease peptone	5 g	
	KH_2PO_4	15 g	
	Na_2HPO_4	7 g	
	NaCl	5 g	
	Dextrose	0.5 g	
	$MgSO_4$	0.25 g	
	Ferric ammonium citrate	0.1 g	
	Sodium pyruvate	0.2 g	
	Distilled water	1 L	

FDA Media Index for BAM (from https://www.fda.gov/food/laboratory-methods-food/media-index-bam)

Pre-enrichment efficacy can be determined by the medium's ability to repeatedly allow for the isolation of the target pathogen at low levels. Factors that influence the efficacy of pre-enrichment media and recovery of *Salmonella* include the composition of the pre-enrichment medium, incubation temperature and length, the presence of competitive bacteria, the extent of *Salmonella* contamination, the extent of stress or injury of the cells, and the biological and chemical characteristics of the food matrix (Daquigan et al., 2016; Jacobson et al., 2017). In

terms of composition, pre-enrichment media typically contains peptones that provide nitrogen, carbon, vitamin, and mineral sources necessary for microbial growth. Some pre-enrichment media, such as BPW and UP broth contain phosphates which improve buffering capacity and sodium chloride to maintain osmotic balance (Taskila et al., 2012). Additionally, UP broth contains tryptone, dextrose, sodium pyruvate, magnesium sulfate, and ferric ammonium citrate which provides additional sources of energy and nutrition to aid in cell proliferation during enrichment (Hammack et al., 2008).

When selecting an efficient enrichment broth for pathogen detection the composition of the food matrix being tested must also be considered as food components can obstruct or delay nutrient transfer in the enrichment culture, potentially affecting the growth of the target pathogen (Taskila et al., 2012). Antimicrobial compounds in foods can influence the effectiveness of a pre-enrichment medium and potentially lead to enrichment failure. Naturally occurring antimicrobials are found in garlic, cumin, soy, and ginger, as well as other foods containing antimicrobial peptides and antimicrobial phytochemicals (Hintz et al., 2015). Antimicrobial components in foods can induce pathogens into a viable but non-culturable (VBNC) cell state. VBNC cells are not detected with culture-based methods and will not grow during enrichment, however they are still virulent, leading to false-negative results (Taskila et al., 2012).

Other food components such as fat, salt, and spices can affect the detection of pathogens and must also be considered. Chen et al. (2010) looked into combatting the effects of inhibitory food components during detection with PCR. It was discovered that with an internal amplification control, identification of potential false-negative samples was possible during the PCR process because the internal amplification control was not adversely affected by the presence of inhibitory compounds. Background microbiota associated with specific food types can also affect pre-enrichment efficiency. Foods such as animal meat and mung bean sprouts, known to carry lactic acid bacteria (LAB), can suppress the growth of enteric bacteria or the resuscitation of injured cells due to a lowered pH (Taskila et al., 2012; Zheng et al., 2015). **Table *2*** summarizes studies that have compared multiple pre-enrichment broths to detect *Salmonella* in various food matrices. These studies have demonstrated that using a specific pre-enrichment medium improved the number of *Salmonella*-positive samples detected in a food sample (Hammack et al., 2001; Jacobson et al., 2017; Zhang et al., 2013). Both UP and BPW were sufficient for the detection of *Salmonella* in leafy greens and herbs. UP broth was effective for orange juice, apple juice, and cucumber, and BPW worked best for the isolation of *Salmonella* from mung bean sprouts (**Table 2)**.

Table 2. Key studies comparing the effectiveness of different pre-*en*richment broths for *Salmonella* isolation.

Food matrix	*Salmonella enterica* Serotypes tested	BAM (2020) recommended enrichment broth	Pre-enrichment broths tested	Most effective enrichment broth	Reference
Pasteurized orange juice	*S.* Gaminara, *S.* Hartford, *S.* Braenderup, *S.* Typhimurium	UP	LB, TT broth, UP broth	UP	(Hammack et al., 2001)
Mung bean sprouts	*S.* Typhimurium, *S.* Montevideo, *S.* Tennessee, *S.* Saintpaul, *S.* Agona, *S.* Newport	UP	BPW; LB, NB, TSB, UP broth,	BPW	(Zheng et al., 2015)
Apple Juice	*S.* Mbandaka, *S.* Newport, *S.* Chester	UP	UP broth, BPW, LB, TT, PBS	UP	(Liao & Fett, 2005)
Fresh cut cucumber	*S.* Mbandaka, *S.* Newport, *S.* Chester	N/A	UP broth, BPW, LB, TT, PBS, and apple juice	UP	(Liao & Fett, 2005)
Pine nuts	N/A, naturally contaminated	N/A	UP broth, mBPW, BAX, BPW, LB	UP, BPW	(Wang et al., 2015)
Romaine lettuce, Italian parsley, cilantro, iceberg lettuce, curl parsley, cilantro, basil, baby spinach	*S.* Aba, *S.* Agona, *S.* Enteritidis, *S.* Gaminara, *S.* Havana, *S.* Javiana, *S.* Michigan, *S.* Montevideo, *S.* Muenchen, *S.* Negev, *S.* Newport, *S.* Saintpaul, *S.* Sandiego, *S.* Thompson, *S.* Typhimurium	UP	BAX, UP broth, mBPW, BPW, LB	UP, BPW	(Jacobson et al., 2017)

UP broth- universal pre-enrichment broth

BPW- buffered peptone water

TT broth- tetrathionate broth

NB- nutrient broth no. 2

BAX- BAX System enrichment media

TSB- tryptone soy broth

PBS- phosphate-buffered saline

mBPW- modified buffered peptone water

LB- lactose broth

2.1.1 Lactose broth

Unless otherwise specified, lactose broth (LB) is the default pre-enrichment medium for detecting *Salmonella* in food, as suggested by BAM Chapter 5, because it does not contain buffering agents (Jacobson et al., 2017). Current detection methods for *Salmonella* in meat substitute products utilize LB as a pre-enrichment medium (FDA, 2020a). While *Salmonella* cannot metabolize lactose, existing background microbes such as *Escherichia coli*, *Klebsiella,* and lactic acid bacteria (LAB) utilize lactose to produce acid, which in turn causes the pre-enrichment pH to become more acidic. A low pH often suppresses the growth of non-acid tolerant microbes present; however, *Salmonella* has a relatively wide range of pH tolerance (3.8-9.5) and can out-compete other microbes under these conditions, making LB slightly more selective than buffered pre-enrichment broths. When the use of LB is followed by selective media and plating, with the validated food matrices outlined in BAM, *Salmonella* levels as low as 1 CFU/ 25 g of the food sample can be detected. However, if pre-enrichment with LB is followed only by qPCR, the LB enrichment alone may not be sufficient to reach the PCR assay detectable levels or 10^3 CFU/mL (Wang et al., 2015). *Salmonella* can achieve pH homeostasis, in which the internal cell pH is maintained at neutral pH despite a low environmental pH, thus protecting the cell from acid stress.

Additionally, *Salmonella* serovar Typhimurium has an acid tolerance response system, which can be activated when pH homeostasis fails or when pH levels are between 5 and 6.5. The acid tolerance response system protects *S.* Typhimurium from protein denaturation due to acid stress at low pH levels (3-4 pH), allowing for an increased likelihood of survival in acidic conditions (Foster & Hall, 1991; Keerthirathne et al., 2016). The acid tolerance response system may be advantageous during enrichment to select for *Salmonella* based on pH survival; however,

it must be done cautiously. False negatives can occur if the *Salmonella* cells become injured or enter the viable but non-culturable state (VBNC) due to significant pH changes.

The BAM Chapter 5 calls for the use of LB for pre-enrichment of *Salmonella* in protein-rich sources, such as meat, rennet casein, and egg-containing products. However, when the effectiveness of LB was compared with other pre-enrichment media, LB was less effective in isolating *Salmonella* from a variety of foods due to the lack of pH stability, resulting in an unfavorable pre-enrichment environment for *Salmonella* proliferation (Hammack et al., 2001; Jacobson et al., 2017; Liao & Fett, 2005; Zheng et al., 2015). Zheng et al. (2015) examined the effectiveness of commercially available selective and non-selective enrichment broths, including LB, nutrient broth no. 2 (NB), tryptone soy broth (TSB), UP broth, BPW, *Salmonella* AD media (AD), BAX System MP media (BAX), ONE broth-*Salmonella* (OB), and selenite broth, to determine the optimal enrichment broth for the detection of healthy and injured *Salmonella* in mung bean sprouts. Zheng et al. (2015) found that for samples enriched with LB at high inoculum levels (10^5 CFU/25g) and 24 h of incubation, the final colony-forming unit (CFU) count was significantly lower from the initial population, resulting in a 0.5 log CFU/ml decrease. Zheng et al. (2015) also found that after 24 h of incubation, the pH of the samples enriched with LB had decreased from 6.73 to 4.2 in low inoculum samples (10^0 CFU/25 g) and 6.63 to 4.17 in highly inoculated samples. After selective enrichment using Rapport Vassiliadis (RV) broth, some *Salmonella* growth was detected in highly inoculated samples, however, no growth occurred in low inoculum samples indicating false-negative results. Zheng et al. (2015) attributed the drop in pH to the production of lactic and acetic acid by lactic acid bacteria (LAB), which is the prevailing microbiota in the mung bean sprouts. In contrast, samples enriched with BPW and ONE broth-*Salmonella* (OB), a commercially available selective enrichment broth, showed little

change in pH. Samples enriched with BPW had a pH decrease from 7.08 to 6.55 in low inoculum (10^0 CFU/25 g) samples after 24 h of incubation and a pH decrease from 7.09 to 6.55 in high inoculum samples (10^5 CFU/25g) after 24 h. Similarly, samples enriched with OB had about a 0.20 pH decrease at both inoculum levels, with a change in pH from 6.99 to 6.76 in low inoculum samples after 24 h of incubation and a decrease from 6.99 to 6.74 in high inoculum samples after 24 h of incubation. Likewise, higher recovery rates of *Salmonella* were observed in both the BPW and OB broths as compared to LB broth. In low inoculum samples enriched in BPW and OB, 4.57 log CFU/mL and 4.71 log CFU/mL, respectively, of *Salmonella* were detected after 24 h incubation. LB resulted in no detection of *Salmonella* after 24 h incubation of low inoculated samples. Recovery rates in highly inoculated samples enriched in BPW and UP resulted in 5.39 log CFU/mL and 6.23 log CFU/mL *Salmonella*, respectively. LB enriched highly inoculated samples obtained plate counts of 3.00 CFU/mL after 24 h incubation, decreasing 0.14 log CFU/mL over the incubation period. The presence of buffers in BPW and OB stabilized the pH, which remained similar at both inoculum levels, likely allowing for sufficient recovery of injured *Salmonella* in comparison to LB-enriched samples (Zheng et al., 2015).

When LB was compared to other pre-enrichment media with acidic foods such as orange juice, apple juice, and apples, LB was unreliable in detecting *Salmonella* (Hammack et al., 2001; Liao & Fett, 2005). Hammack et al. (2001) examined the recovery of *Salmonella* in orange juice with various pre-enrichment media, including LB, Tetrathionate (TT) broth, and UP broth. Hammack et al. (2001) found that the number of *Salmonella*-positive samples recovered using LB was significantly less compared to orange juice samples enriched with UP broth. It was hypothesized that due to the more acid-tolerant microflora present in orange juice, the lactose

was quickly converted to acid, preventing injured *Salmonella* from resuscitating and resulting in false-negative results (Hammack et al., 2001). These findings suggest that while *Salmonella* is relatively acid-tolerant, foods associated with acid-producing microbiota cause the pre-enrichment pH to drop drastically. A lower pH, prevents the rehabilitation of injured *Salmonella*, affecting the detection of *Salmonella,* and potentially causing false negatives.

2.1.2 Buffered peptone water

Buffered peptone water (BPW) is compliant with both the International Organization for Standardization (ISO) and FDA/BAM, making it one of the most widely used non-selective enrichment broths for *Salmonella* (Taskila et al., 2012). The peptones in BPW provide nitrogen, carbon, vitamins, and minerals; sodium chloride preserves osmotic balance; and the phosphates (disodium phosphate and mono-potassium phosphate) provide buffering capacity, allowing for a more stable pH during enrichment (Taskila et al., 2012). BAM Chapter 5 calls for the use of BPW as pre-enrichment for the isolation of *Salmonella* from mangoes and animal feed. However, studies have also been conducted to determine the efficacy of BPW with other food matrices. For example, Zheng et al. (2015) determined BPW to be the most efficient pre-enrichment broth, in terms of effectiveness and cost, for the isolation of *Salmonella* from mung bean sprouts in comparison to UP, LB, and several other commercially available enrichment broths.

Meat analogs may perform well when pre-enriched with BPW due to the similarities with animal feed in terms of protein sources. Animal feed commonly contains plant ingredients such as soybean meal and wheat grain, which are also common sources of protein in meat analogs. Understanding the effectiveness of BPW in isolating *Salmonella* from these animal feed materials can give insight into its effectiveness in other plant-protein products. Koyuncu and Häggblom (2009) compared cultural methods for detecting *S. enterica* in animal feed material,

including soybean meal and wheat grain. Using BPW for pre-enrichment, the pH remained stable, demonstrating BPW buffering capacity. During pre-enrichment, pH is a good indication of cell recovery, as a low pH value during pre-enrichment can affect the viability of bacteria, therefore having a stable pH will maintain cell viability. Koyuncu and Häggblom (2009) measured the pH after enrichment with BPW for each animal feed tested and found that the pH remained close to neutral for all animal-feed material, with soybean meal having a pH value of 5.2 and wheat grain a pH of 6.5. According to EN ISO 6579:2002, the pH during enrichment with BPW should remain above 4.5. In the BAM, a pH adjustment to 6.8 ± 0.2 before incubation is recommended when using BPW (FDA, 2020a). The findings of Koyuncu and Häggblom (2009) indicate that BPW has a sufficient buffering capacity to successfully sustain a favorable pH during pre-enrichment of soy and wheat grain material.

While BPW is a widely used pre-enrichment broth, inconsistencies amongst commercial brands of BPW in their ability to recover *Salmonella enterica* have been found (Margot et al., 2015). Baylis, MacPhee, & Betts (2001) compared the efficacy of two different commercial preparations of BPW in the recovery of heat-injured *Salmonella* and found that one brand recovered significantly more heat-injured cells in low levels of pure culture compared to the other brand, despite the second brand having a higher buffering capacity. Baylis et al. (2001) hypothesized that the difference between brands may result from high levels of phosphate in the second brand, which can increase levels of toxic oxygen species after autoclaving. These findings are important to consider when using commercially acquired BPW; however, Baylis et al. (2001) also found little difference amongst the two BPW brands in the recovery of *Salmonella* from milk powder, cottage pie ready-meal, cooked turkey, uncooked minced beef, and sprouts. The results of Baylis et al. (2001) suggested the BPW success depended more on the food source

which may have been related to competitive microbiota native to the food type. In most of the foods tested, the final cell concentration reached between 4 log CFU/ml to 8 log CFU/ml despite the presence of native bacteria. In contrast, raw bean sprouts had high background microflora of 6 log CFU/ml before incubation, leading to greater levels of background microflora being detected compared to *Salmonella.* Of the serotypes tested, only *S.* Typhimurium and *S.* Enteritidis were detected, whereas serotypes *S.* Agona, *S.* Newport, *S.* Heidelberg, and *S.* Infantis could not be recovered from the raw bean sprouts enriched in BPW after 24 h of incubation due to failure to reach detectable levels (Baylis et al., 2001). These findings further emphasize the importance of understanding food composition and the biological impact of natural microflora on the success of pre-enrichment.

2.1.3 Universal pre-enrichment broth

Universal pre-enrichment (UP) broth, like BPW, contains buffering phosphates and sodium chloride for osmotic balance. Additionally, UP broth contains ingredients such as dextrose as an energy source, tryptone as an amino acid source, as well as ferric ammonium citrate, sodium pyruvate, and magnesium sulfate which aid in the proliferation of injured and uninjured cells (Hammack et al., 2008). Several researchers have found greater success in isolating *Salmonella* using UP broth for pre-enrichment compared to other commercially available pre-enrichment media, including LB and BPW, for specific food matrices such as orange juice, apple juice, and cucumber (Hammack et al., 2001; Jacobson et al., 2017; Liao & Fett, 2005; Wang et al., 2015). These findings align with current BAM Chapter 5 methods which call for the use of UP broth for various low acid/acidic foods, including tomatoes, mamey pulp, fresh leafy greens, herbs, sprouts, lactic casein, vegetables, orange juice, apple cider, apple juice and cantaloupes (FDA, 2020a). Based on the BAM, UP broth is typically used for fresh produce and foods from plant sources.

When Hammack et al. (2001) examined the effectiveness of various pre-enrichment broths in isolating *Salmonella* from orange juice, UP broth was the most effective, noting that the presence of sodium pyruvate increased the recovery of *Salmonella* compared to LB. Certain ingredients in UP broth, specifically sodium pyruvate, magnesium sulfate, and ferric ammonium citrate, have all been shown to aid injured cells in resuscitation, an essential aspect of pre-enrichment (Hammack et al., 2008). A subsequent study conducted by Hammack et al. (2008) examined the efficacy of UP broth, LB, and BPW in identifying *Salmonella* Typhi in mamey pulp. While UP broth was the most effective pre-enrichment medium, pH did not play a role in the effectiveness of UP broth. This was determined because LB, as a non-buffered enrichment had similar results to BPW, a buffered enrichment. Based on these results, Hammack et al. (2008) aimed to examine which ingredients (i.e., ferric ammonium citrate, magnesium sulfate, or sodium pyruvate) would affect the recovery of *S.* Typhi. While the broth containing all three ingredients was most effective, UP broth without ferric ammonium citrate was more productive than UP broth containing ferric ammonium citrate for the recovery of *S.* Typhi in mamey pulp. Hammack et al. (2008) found that the inhibition of *S.* Typhi due to ferric ammonium citrate was only in samples containing mamey and at low levels of *S.* Typhi. These findings led Hammack et al. (2008) to hypothesize that inhibition was most likely due to background microflora specific to mamey pulp outcompeting *S.* Typhi with the nutrients obtained from ferric ammonium citrate. Similar studies have not been conducted with non-typhoidal *Salmonella,* however, the Hammack et al. (2008) study further demonstrates the importance of understanding the effects of background microflora typical to food matrices concerning the efficacy of the pre-enrichment broth.

Overall, UP broth is advantageous as it contains ingredients such as ferric ammonium citrate, magnesium sulfate, and sodium pyruvate known to provide nutrients for the resuscitation of injured cells (Hammack et al., 2008). Additionally, phosphate buffers in UP broth aid in maintaining the pH which can increase the success of *Salmonella* isolation. As with most pre-enrichment media, UP broth may be disadvantageous when ingredients such as ferric ammonium citrate allow for microbes that are native to the food matrix to outcompete *Salmonella.*

Table 3. Current uses, compositional advantages and disadvantages, and cost of BPW, LB, and UP pre-enrichment

Pre-enrichment broth	Lactose broth	Buffered Peptone Water	Universal pre-enrichment broth
Current uses	Default pre-enrichment broth. Egg and egg-containing products, powdered mixed (cake, cookie, etc.), infant formula, rennet casein, sodium caseinate, cheese, dough, prepared salads, fruits, nut meats, crustaceans, fish, coconut, gelatin, frog legs, rabbit carcasses, guar gum, dog chews, alfalfa seeds, mung beans, meats and meat substitutes.	Mangoes, cabbage, animal food (dry dog and cat food, cattle feed, horse, poultry, and swine feed)	Lactic casein, vegetables, orange juice, apple cider, and apple juice, cantaloupes, tomatoes, mamey pulp. leafy green vegetables, herbs, sprouts (baby spinach, iceberg lettuce, Romaine lettuce, Spring mix, cilantro, curly parsley, culantro, Italian parsley, alfalfa, mung bean, clover, radish and broccoli sprouts)
Compositional advantages	**Lactose:** acts as a selective agent when lactose fermenting bacteria are present due to lowered pH **Peptone:** source of nitrogen, carbon, vitamins, and minerals **Beed Extract:** added nutrients	**Phosphate buffers:** pH stabilization during enrichment **Peptone:** source of nitrogen, carbon, vitamins, and minerals **Sodium chloride:** Offers osmotic balance	**Phosphate buffers:** pH stabilization during enrichment **Peptone:** source of nitrogen, carbon, vitamins, and minerals **Sodium chloride**: Offers osmotic balance **Dextrose & Tryptone:** Energy source **Sodium pyruvate, magnesium sulfate, and ferric ammonium citrate:** Aid in injured/un-injured pathogenic cell proliferation
Compositional disadvantages	**Lactose:** fermentation of lactose decreases pH less than 3.8 can lead to *Salmonella* cell injury or death	**Phosphate buffers:** neutral pH supports the growth of competitive microbes leading to competition for nutrients	**Phosphate buffers:** neutral pH supports the growth of competitive microbes leading to competition for nutrients
Cost per package	$84.00/ 500 g LB + $107.10/100 mL Triton X-100	$100.80/ 500 g BPW	$79.90/ 500 g UP
Grams per sample	2.93 g LB / 225 mL + 2.25 mLTriton X-100/ 225 mL	4.5 g BPW / 225 mL	8.55 g UP/ 225 mL
Cost per sample	$2.91 ($0.50/225 mL LB + $2.41/2.25 mL Triton X-100)	$0.76 (per 225 mL BPW)	$1.36 (per 225 mL UP)
References	(FDA, 2020a; Hammack et al., 2001; Hammack et al., 2008; Neogen, 2021; Taskila et al., 2012; Wang et al., 2015)		

Pricing obtained from Fischer Scientific website and reported in USD. All LB (Catalog No. 50-201-5168), BPW (Catalog No. 50-201-5162), and UP (Catalog No. 50-2-105279) prices are for NEOGEN manufactured products. Triton X-100 listed price is for products manufactured by Millipore Sigma (Catalog No. M1122980101**)**

2.2. *Salmonella enterica* in plant protein

The CDC estimates that *S. enterica* is responsible for 1.35 million infections, 25,500 hospitalizations, and 420 deaths in the United States each year (CDC, 2020). Non-typhoidal *S. enterica* results in salmonellosis, which is associated with fever, abdominal pain, vomiting, and diarrhea. Typhoidal *Salmonella* is responsible for typhoid fever, which, although less common than salmonellosis, is life-threatening and is associated with fever, headache, lethargy, and anorexia (Knolder & Elfencein, 2019). *S. enterica* is a rod-shaped, Gram-negative, facultative anaerobe bacteria (FDA, 2012; Knolder & Elfencein, 2019). Additionally, *Salmonella* is fairly acid-tolerant and has a wide temperature growth range (5- 47 °C), allowing it to survive at refrigerated temperatures (Smadi et al., 2012). These characteristics are important when considering *Salmonella's* survivability in food. As a facultative anaerobe, *Salmonella* prefers an aerobic environment but can survive without oxygen. Similarly, its broad temperature and pH range increase survivability in various foods, including cold storage items. Meat analog products are often sold frozen (e.g., Morningstar Farms™, Gardein™, and Quorn™) or refrigerated (e.g., Impossible Foods™ and Beyond Meat™); therefore, if kill steps during processing by manufacturers are ineffective, there is a possibility of *Salmonella* surviving during refrigerated or frozen storage. While cooking instructions provided by brands like Beyond Meat™ recommend consumers cooking the product to reach an internal temperature of 165°F, if the consumers fail to cook the plant-based burger to an internal temperature of 160-165°F (71-74°C), *Salmonella* can potentially go on to cause illness (FDA, 2018a).

Salmonella is typically associated with animal products, specifically poultry and eggs, as its reservoirs include the intestinal tracts of warm and cold-blooded animals. However, *Salmonella* has also been associated with non-animal sources, including nuts, raw flour, sprouts, fruits, and vegetables (Andino & Hanning, 2015; CDC, 2019). Contamination of produce items can occur at

any stage (cultivation, handling, processing, and preparation steps) during the farm to fork process (Fatica & Schneider, 2011; Jacobson et al., 2017). *Salmonella* is spread by the fecal-oral route; before harvesting, crops are susceptible to contamination from fecal matter, animal contact, or water containing animal feces (Andino & Hanning, 2015; Fatica & Schneider, 2011). Nsoesie, Kluberg, and Brownstein (2014) found that about 32% of the foods implicated in outbreaks in the CDC Foodborne Outbreak Online Database (FOOD) were from plant sources (fruits, nuts, and vegetables), while contaminated animal products accounted for the rest. Contaminated irrigation water is a major concern for produce safety as it can act as a vehicle for contaminating plant surfaces, as well as the surrounding soil (Fatica & Schneider, 2011). Sprouts, a member of the Leguminosae family, are typically grown in water, soil, or hydroponics; if clean water or soil is not maintained, the risk of contamination increases (Reed et al., 2018). Additionally, the extended sprouting period at ambient temperatures and high humidity can encourage pathogen growth in germinating legume seeds, in turn increasing the risk of illness for those consuming sprouts (Fu et al., 2008; Landry et al., 2014)

The *S. enterica* species contains five subspecies and about 2600 serotypes. While outbreaks of *Salmonella* have not specifically been related to meat analog products in the United States, several serotypes have been associated with ingredients used in meat analogs or products with similar ingredients as meat analogs. *S. enterica* serovar Enteritidis and *S.* Typhimurium have been associated with over half of the salmonellosis cases in the United States and have both been associated with plant protein sources and plant protein-containing products (Fatica & Schneider, 2011). For example, a 2015 outbreak of *Salmonella* Enteritidis in mung bean sprouts infected 115 people across 12 different states and resulted in 19 hospitalizations (FDA, 2015a). Mohle-Boetani et al. (2009) investigated seven outbreaks of *S.* Enteritidis in raw mung bean

sprouts that occurred between 2000-2002 in the Netherlands, Canada, and the United States. Mohle-Boetani et al. (2009) noted that no *S.* Enteritidis outbreaks had been associated with mung bean sprouts before 2000, and the majority of the outbreaks occurred over three years as a result of sprout growers' failure to follow the FDA guidelines for seed disinfection. A 2001 outbreak in Hawaii linked the mung bean sprout contamination to spent irrigation water, which tested positive for *Salmonella.* It is important to note that in all cases investigated by Mohle-Boetani et al. (2009), mung bean sprouts were consumed raw. While mung bean is commonly used in meat analogs as a protein source, the raw mung beans are typically processed into mung bean flour and pasteurized when obtaining mung bean protein isolate. The pasteurization step serves to inactivate *Salmonella,* making mung bean protein less likely to cause illness compared to raw mung bean sprouts (FDA, 2016)

Animal feed is a common source for *Salmonella* contamination. Soy protein is frequently used as a feed ingredient and it is also one of the most common plant proteins used in the top-selling meat analogs in the U.S. (**Table 4**). Wierup and Häggblom (2010) sampled plant proteins from five different Swedish feed meal companies and found that soybean meal products were most often contaminated, with 14.6% of the samples being infected. Of the serotypes isolated from the soybean meal tested, *S.* Agona, *S.* Typhimurium, *Salmonella* Infantis, and *Salmonella* Kentucky were also among the ten most common isolates of human cases of salmonellosis in the European Union (EU). *S.* Agona was also associated with a 2008 outbreak in wheat puff cereal, which was associated with 28 cases across 15 states (CDC, 2008). A major ingredient in this cereal was wheat, which is another common ingredient used in meat analogs. While the report did not list the cause of the *S.* Agona contamination, wheat is highly susceptible to pathogenic contamination throughout the production process and may have played a role in the outbreak.

Understanding the risk of *Salmonella* contamination in plant proteins such as mung bean, wheat, and soy, all of which are used in meat analogs, confirm the need for effective and efficient methods to detect *Salmonella* in these plant-protein-containing products.

2.3. Key protein ingredients in meat analogs

Meat analogs are products that utilize plant- or fermentation-based (mycoprotein) proteins to mimic the texture, flavor, and appearance of traditional meat products, such as burgers and patties (Pietsch et al., 2017; Sha & Xiong, 2020). Key ingredients in meat analogs to mimic the texture, flavor, and appearance of traditional meat products include plant proteins, hydrocolloids, and fats. The nutritional content and functional properties influence which plant-proteins are used by meat analog manufacturers, contributing to variation in plant proteins being used in meat analogs. Along with satisfying the ethical needs of consumers, meat analog products are nutrient dense as they often contain essential amino acids, are low in saturated fat, and are cholesterol-free (Samard & Ryu, 2019). Understanding both the chemical and microbiological components associated with meat analog products and their ingredients can aid in the proper selection of pre-enrichment broth to recover pathogens.

Table 4. Ingredients found in major meat-analog burgers sold in the United States (major protein sources are indicated with boldface).

Brand	Burger	Ingredients List
Morningstar Farms™	Morningstar Farms® Meat Lovers Vegan Burgers	Water, vegetable oil (corn, canola, and/or sunflower oil), **wheat gluten, soy protein isolate, soy flour** contains 2% or less of natural flavor, methylcellulose, cornstarch, salt, cooked onion, and carrot juice concentrate, sunflower oil, spices, garlic powder, onion powder, yeast extract, tomato paste (tomatoes), xanthan gum
	Morningstar Farms® Veggie Lovers Vegan Burgers	Water, onions, carrots, mushrooms, cooked **brown rice** (water, brown rice), celery, vegetable oil (corn, canola, and/or sunflower), brown **lentils** (water, lentils), spinach, **soy flour, soy protein isolate**, contains 2% or less of pumpkin seeds, sunflower seed, spices, methylcellulose, sweet potato powder, salt, red bell peppers, sugar, green bell peppers, potato starch, dried garlic, yeast extract, konjac flour, xanthan gum, tomato powder, natural flavor, garlic powder)
	Morningstar Farms ®Grillers Original Burgers	Water, **wheat gluten**, **soy flour**, vegetable oil (corn, canola and/or sunflower), **egg whites**, calcium caseinate, cornstarch, contains 2% or less of onion powder, soy sauce powder (soy sauce [soybeans, salt, wheat]), methylcellulose, cooked onion, and carrot juice concentrate, salt, natural flavor, **soy protein isolate**, garlic powder, spices, sugar, gum acacia, whey, yeast extract, xanthan gum, potato starch, tomato paste, onion juice concentrate
	Morningstar Farms® Cheezeburger	Water, vegetable oil (corn, canola, and/or sunflower oil), **wheat gluten**, cheddar style vegan cheeze (water, modified corn starch, coconut oil, tapioca starch, natural flavor, tricalcium phosphate, canola oil, glycerine, salt, sodium citrate, **pea protein,** cultured dextrose, xanthan gum, sunflower lecithin, color added), **soy protein isolate, soy protein concentrate,** contains 2% or less of natural flavor, methylcellulose, cornstarch, salt, cooked onion, and carrot juice concentrate, spice, garlic powder, onion powder, tomato paste, xanthan gum
Gardein™	Ultimate Plant-Based Burger	Water, **textured pea protein, textured wheat protein** (wheat gluten, wheat starch), palm oil, coconut oil, **vital wheat gluten**, contains 2% or less of: methylcellulose, malt extract, natural flavors, garlic powder, organic sunflower oil, sea salt, onion powder, beet juice, citric acid, ascorbic acid
	Ultimate Beef-less Burger	Water, **textured wheat protein** (wheat gluten, wheat flour, malted barley extract), **vital wheat gluten**, **soy protein concentrate**, onions, expeller pressed/canola oil, **soy protein isolate,** organic **ancient grain flour** (organic LAMUT Khorasan Wheat Flour, organic amaranth flour, organic millet flour, organic quinoa flour), modified vegetable gum, yeast extract, dehydrated garlic, onion powder, malted barley extract, organic cane sugar, sea salt, natural flavors, potato starch, spices, vinegar, **pea protein,** carrot fiber, beetroot fiber
Beyond Meat™	Beyond Burger®	Water, **pea protein**, expeller-pressed canola oil, refined coconut oil, **rice protein**, natural flavors, cocoa butter, **mung bean protein**, methylcellulose, potato starch, apple extract, pomegranate, extract, salt, potassium chloride, vinegar, lemon juice concentrate, sunflower lecithin, beet juice extract (for color)

Impossible Foods ™	Impossible™ Burger	Water**, Soy Protein Concentrate**, Coconut Oil, Sunflower Oil, Natural Flavors, 2% Or Less Of: **Potato Protein**, Methylcellulose, Yeast Extract, Cultured Dextrose, Food Starch Modified, Soy Leghemoglobin, Salt, **Soy Protein Isolate**, Mixed Tocopherols (vitamin E), Zinc Gluconate, Thiamin Hydrochloride (vitamin B1), Sodium Ascorbate (vitamin C), Niacin, Pyridoxine Hydrochloride (vitamin B6), Riboflavin (vitamin B2), Vitamin B12
Quorn™	Quorn™ Vegan Meatless Spicy Patties	**Mycoprotein** (54%), **wheat flour** (wheat flour, calcium carbonate, iron, niacin, thiamine), sunflower oil, water, **potato protein**. contains 2% or less of pea fiber, wheat starch, **wheat gluten**, calcium chloride, calcium acetate, yeast extract, chili flakes, parsley, salt, yeast, garlic powder, onion powder, tomato powder, cayenne pepper, white pepper, carrageenan, sodium alginate, rice flour, natural flavors, paprika extract, sage, sugar, leavening (ammonium bicarbonate).
	Meatless Gourmet Burgers	**Mycoprotein (37%), egg white, wheat flour,** onion, palm oil, canola oil, **milk protein concentrate.** contains 2% or less of natural flavor, roasted barley malt extract, salt, maltodextrin, dextrose, calcium chloride, calcium acetate, potassium chloride, citric acid, sugar, potato dextrin, yeast, coconut oil, black pepper.
BOCA™	Boca Extra-Large All-American Veggie Burgers	Water, **soy protein concentrate**, **reduced-fat cheddar cheese** (pasteurized part-skim milk, cheese culture, salt enzymes, annatto [color], vitamin palmitate), corn oil, **wheat gluten**, contains less than 2% of the following: caramel color, methylcellulose, **soy protein**, dried onion, cheese powder (cheddar cheese [milk, cheese culture, salt, enzymes], cream, salt, sodium phosphate, lactic acid), salt, natural flavor (non-meat), **hydrolyzed soy protein**, disodium inosinate, and disodium guanylate, sesame oil, spices and herbs, dried garlic, mannitol**,**
	Boca Original Vegan Veggie Burger	Water, **soy protein concentrate**, **wheat gluten**, contains less than 2% of salt, methylcellulose, corn oil, onion powder, garlic powder, caramel color, yeast extract, malt extract (contains gluten), natural flavor (non-meat), spices.

2.3.1. Plant proteins used in meat analogs

2.3.1.1. Legumes: soy bean, mung bean, and pea protein

Legumes, including soy beans, mung beans, and peas, are commonly used plant protein sources in meat analog products. In addition to protein, these ingredients are also sources of fiber, B-12 vitamins, iron, copper, magnesium, and carbohydrates. Legumes are low in saturated fat and cholesterol-free (Polak et al., 2015). The major constituents of legume seeds include protein, oil, starch, fiber, and sucrose. The concentration of these proximates differs depending on the legume source. For example, soybean contains higher protein (35-42% of seed weight), oil (17-21% of seed weight), and fiber (20% of seed weight) compared to mung bean (protein 23% of seed weight, oil 1.2% of seed weight, and fiber 7% of seed weight) and pigeon peas (protein 20% of seed weight, oil 1.2% of seed weight, and fiber 7% of seed weight). However, mung bean and pigeon pea seed contain higher starch (about 45% of seed weight) compared to soybean (1.5% of seed weight) (Kumar & Pandey, 2020). Differences in the proximate composition may influence the legumes' contribution to nutrition and function in meat analogs.

2.3.1.1.1. Soybean protein

Soybean is the most nutritionally rich legume. It contains all the essential amino acids and is a good source of unsaturated fatty acids, B vitamins, fiber, and phytochemicals such as isoflavones (Rizzo & Baroni, 2018). A disadvantage associated with soy is the likelihood of allergenicity, as it is the seventh most common allergen in the U.S. and is therefore avoided by many consumers and manufacturers to prevent the risk of allergen hazards (FDA, 2020b). The prevalence of soy allergies is reported to be about 0.6% of U.S adults aged 18-60 (Gupta et al., 2019).

Soy isoflavones have both positive and potentially negative impacts on health (Barnes et al., 2000; Rizzo & Baroni, 2018). Isoflavones have antioxidant properties which reduce the long-

term risk of cancer, by preventing DNA damage due to free radicals, and reduce the risk of cardiovascular disease by decreasing low-density lipoproteins (LDL) and cholesterol levels (Djuric et al., 2001; NCI, 2017; Patel et al., 2001; Zaheer & Akhtar, 2017). A meta-analysis study by Anderson et al. (1995) found that consuming 47 g of soy daily decreased total cholesterol by 9.3%. However, all analyzed studies cited high consumption levels of soy (17-124 g/day) compared to typical consumption levels of 10-12 g of soy a day for the average vegan, and about 5-6 g a day for non-vegans (Rizzo & Baroni, 2018). Alekel et al. (2000) reported that consumption of 80 mg soy protein isolates with isoflavones reduced bone loss in premenopausal women.

In contrast, there are some potential health concerns associated with the consumption of soy isoflavones. Specifically, the phytoestrogen isoflavones genistein and daidzein have been associated with inducing cancer in the reproductive organs of rodents (Murata et al., 2004). Murata et al. (2004) suggest that the combination of the isoflavones influence on cell proliferation and metabolites contribute to increased oxidative DNA damage which may induce tumor progression. While these findings may be a cause for concern, studies have not linked soy consumption with tumor development in humans (Okekunle et al., 2020). Additionally, in contrast to Murata et al. (2004) study, genistein and daidzein may have chemo-preventive properties (Hwang & Choi, 2015; Lamartiniere et al., 2002). Lamartiniere et al. (2002) reported genistein as protecting against breast and prostate cancer in rats. These conflicting studies demonstrate these findings may be case sensitive and outside factors such as gender, age, diet, and lifestyle may influence the findings. Additionally, other factors such as genetic predisposition, a sedentary lifestyle, alcohol abuse, radiation to the chest, etc., are better-reported risk factors for breast cancer (NBCF, 2021).

Soy isoflavones have antimicrobial properties against *Listeria monocytogenes* and *Escherichia coli* (Dhayakaran et al., 2015). Specifically, growth of *L. monocytogenes* was inhibited by 10 μg/mL soy isoflavones and *E. coli* biofilms were inhibited by 100 μg/mL soy isoflavones. However, the researchers did not examine the antimicrobial effect against *Salmonella*. The processing of soy-based foods can alter the composition and nutrient content, potentially affecting the activity of these proteins. In meat analogs specifically, soy flour is used to obtain textured soy protein (TSP) through extrusion and cooking. Samard and Ryu (2019) examined the physiochemical characteristics of meat analogs composed of TSP and wheat gluten and the effects of heat-treatments on moisture and amino acid content of the TSP. Samard and Ryu (2019) reported increased TSP moisture content compared to raw material correlated with a reduction in total amino acid content (Samard & Ryu, 2019). Murphy et al. (2002) solvent extracted soy isoflavones in soy-based foods, and reported that TSP has more acetyl forms of isoflavones known as glucosides (50% malonyl-β-glucosides with 32% β-glucosides and 20% acetyl- β-glucosides forms), which are not as readily absorbed compared to the isoflavones found in fermented soy foods, such as tempeh and miso. Murphy et al. (2002) also noted that soy foods that are highly processed could result in a maximum 80% loss of isoflavones. Isoflavone loss could, in turn, decrease the antimicrobial activity in TSP. In addition to isoflavones, soybeans contain long-chain peptides, which can act as an antimicrobial against Gram-negative and Gram-positive bacteria. As a result, soy products have some of the highest diversity of antimicrobial compounds compared to other legumes (Pina-Perez & Perez, 2018).

Methylated soy protein has demonstrated antimicrobial activity against *Listeria* and *Salmonella* (Sitohy et al., 2013). Sitohy et al. (2013) found that after 6-12 h of incubation at 37 °C, the methylated soy proteins extracted from soy protein isolates inhibited 91% of *S.*

Enteritidis growth with a minimum inhibitory concentration of about 100 μg/mL. While the antimicrobial potential of soybeans is advantageous from a food safety perspective, it may affect the pre-enrichment and detection of *Salmonella* in meat analog samples. If antimicrobials present during pre-enrichment are active against bacteria such as *L. monocytogenes* and *E. coli*, *Salmonella* will better proliferate in the pre-enrichment broth due to less competitive conditions. However, if the antimicrobials are active against the target pathogen during pre-enrichment, soy may result in inhibition of *Salmonella* growth, potentially affecting the ability to reach detectable levels, isolate, and detect *Salmonella* in meat analog samples containing soy. Therefore, meat analogs sampled from brands such as MorningStar Farms™ and Impossible Foods™ may require a more nutrient-rich pre-enrichment to combat the antimicrobial nature of soy and soy proteins.

Table 5. Antimicrobial activity associated with plant-proteins in meat analogs and their impact on *Salmonella* pre-enrichment

Protein source	**Natural antimicrobials present**	**Antimicrobial activity**	**Potential impact on pre-enrichment**	**References**
Soy protein extracts in soy flour; Soy protein in soybean seed	Soy isoflavones, long-chain peptides, methylated soy protein	*100ug/mL MIC[1] against L. monocytogenes[2] Salmonella* Enteritidis, *E. coli[3]*	Antimicrobial activity may decrease competition and lead to difficulty in isolating *Salmonella.*	(Dhayakaran et al., 2015; Pina-Perez & Perez, 2018; Sitohy et al., 2013)
Mung bean seed	Polyphenolic compounds with elicitors (fish protein hydrolysates, lactoferrin, oregano extract) Mung bean protein with bioactive pomegranate peel compounds	*200 ul mung bean phenolic compounds led to 0.4 cm zone of inhibition (no elicitors present) of Helicobacter pylori*	Mung bean protein itself has no proven antimicrobial activity; mung bean protein with elicitors (Oregano, fish protein hydrolysates, lactoferrin) or pomegranate peel could decrease competition during enrichment	(Moghadam et al., 2020; Randhir et al., 2004)
Pisum sativum L.	Pea peptides	*72-108 ug/mL MIC against S. aureus[4], P. aeruginosa[5], E. coli[2],* and *S.* Typhi[6]	Antimicrobial activity may decrease competition and lead to difficulty in isolating *Salmonella*..	(Rehman & Khanum, 2011)
Wheat	N/A	N/A	N/A	N/A
Mycoprotein	N/A	N/A	N/A	N/A
Potato tubers (*Solanum tuberosum L.)*	Proteins: Potide-G, AFP-J, Potamin-1, and PG-2	*1000-1250 ug/m MIC against S. aureus[4], Salmonella E. coli[3]*	Antimicrobial activity may decrease competition and lead to difficulty in isolating *Salmonella.*	(Bártová et al., 2019; Jin et al., 2008)

1 . Minimum inhibitory concentration; 2. Listeria monocytogenes; 3. Escherichia coli; 4. Staphylococcus aureus; 5. Pseudomonas aeruginosa; 6. Salmonella Typhi

While soy is a great protein source nutritionally, soy has been associated with increased microbial growth and accelerated spoilage in extended beef products. Meat-extenders, such as soy, are non-animal protein sources that provide dietary fiber, reduced caloric content, and sometimes enhance flavor perception to beef and other meat products (Wong et al., 2019). Keeton and Melton (1978) examined the factors affecting microbial growth in beef products containing varying amounts of TSP and found that as the concentration of TSP increased, total coliform counts increased, resulting in faster spoilage of the beef and soy patties. Keeton and Melton (1978) mentioned that while it was likely the TSP extender was the cause of the increased colony count, the increase in microorganisms associated with TSP might have also been due to the greater variety of raw material in the soy extended beef patties. While coliforms are typically a concern for spoilage, recognizing the increased number of coliforms associated with TSP is important as it may lead to increased competition for nutrients during pre-enrichment, potentially resulting in lower detection levels of the target pathogen. *Klebsiella pneumoniae* is a Gram-negative, lactose fermenting pathogenic bacteria known to be native to soybean. The potential presence of *Klebsiella pneumoniae* in soy-based foods may affect pre-enrichment with LB because the production of lactic acid would decrease the pH, thus affecting the rehabilitation and isolation of *Salmonella* from meat analogs containing soy (Wang et al., 2015).

2.3.1.1.2. Mung bean protein

Mung bean is another common protein source used in meat analogs such as the Beyond Burger®. Mung bean is rich in protein (about 20% to 30%); however, it is an incomplete protein source, with methionine and cysteine being the limiting essential amino acids (Pina-Perez & Perez, 2018; Tang et al., 2014; Xia et al., 2020; Yi-Shen et al., 2018). In combination with cereals, mung beans can offer high-quality protein and improved digestibility with reported

PDCAAS and DIASS scores between 50-56% and 37-53%, respectively (FDA, 2016; Yi-Shen et al., 2018). Additionally, mung bean protein is often utilized in meat analogs for its gelling potential to improve the structure and texture of meat analogs (Yi-Shen et al., 2018). Brishti et al. (2017) compared the least gelation concentration (LGC) of mung bean and soy protein isolates and found that mung bean had a slightly smaller LGC (12%) in comparison to soy (14%). LGC describes the minimum amount of protein required for gel to form, therefore a lower LGC indicates a greater gelling ability. Compared to soy, mung beans are rich in polyphenolic compounds. Randhir, Lin, and Shetty (2004) reported that mung bean sprouts with added elicitors (lactoferrin and oregano extract) inhibited the growth of *Helicobacter pylori.* While antimicrobial activity of stimulated mung bean sprouts has been observed, mung bean protein by itself does not exhibit significant antimicrobial activity. Moghadam et al. (2020) examined the antimicrobial effects of edible antioxidant films made from mung bean protein and pomegranate peel, and reported that the mung bean and pomegranate films inhibited *L. monocytogenes* and *E. coli*. However, it is important to note that the control sample containing only mung bean protein had no reported antimicrobial activity, and the antimicrobial activity of the mung bean/pomegranate films increased as pomegranate concentration increased (Moghadam et al., 2020).

In terms of microbiota, mung beans have been associated with lactic acid bacteria (Zheng et al., 2015). As previously mentioned in section **2.1**, the presence of lactic acid bacteria (LAB) during pre-enrichment with LB produces lactic acid, which can interfere with the detection of *Salmonella* in mung bean sprouts (Zheng et al., 2015). Biofilms are complex microbial ecosystems formed by one or more microbial species immersed in an extracellular matrix composed of polysaccharides, proteins, or exogenous DNA (Galié et al., 2018). Biofilms in both

food or object's surface are a concern in the food industry as they are difficult to remove from food and more resistant to processing procedures (Fett & Cooke, 2003). Fett and Cooke (2003) examined biofilms native to mung bean sprouts to better understand mung bean biofilm composition. The biofilms were especially abundant on the cotyledon (embryonic leaves of seed plants) surfaces of the mung bean sprouts and a variation of rod-shaped bacteria, cocci bacteria, and yeast was observed. The presence of these diverse biofilms can be a cause of concern for meat analog products that utilize mung bean protein. Additionally, biofilms have the potential for increasing competition during pre-enrichment of meat analog samples, interfering with the detection of *Salmonella* in meat-analogs containing mung bean. While biofilms are difficult to remove from food sources, sufficient processing techniques of mung bean proteins (seed disinfection and pasteurization), and proper cleaning and sanitizing of meat analog production facilities should reduce these biofilms to safe levels. Additionally, the highly processed mung bean protein used in meat analogs would not be expected to contain viable microflora.

2.3.1.1.3. Pea protein

Pea protein is another common legume used in meat analogs for structural and nutritional qualities. Proximate composition varies depending on the pea variety, with the composition majority being 42-53% starch and 21-26% protein (Millar et al., 2019). Pea protein can increase viscoelasticity which, when combined with other protein sources, offers a desirable texture to meat analog consumers. Yuliarti et al. (2021) found that while pea protein increased the hardness, chewiness, and viscoelasticity of meat analogs containing pea and wheat protein when measuring sensory attributes, participants most enjoyed the texture of meat analogs with 4% pea protein and 13% wheat protein. Pea protein is more likely to be used in smaller amounts or in combination with other plant-proteins in meat analogs for this reason. Pea protein also has antimicrobial activity, with reported inhibitory effects against *Staphylococcus aureus,*

Pseudomonas aeruginosa, *E. coli*, and *S.* Typhi. Rehman and Khanum (2011) examined the effectiveness of pea peptide isolated from *Pisum sativum* (pea) seeds against several bacterial pathogens and found the pea peptides from the seed and pod were most effective against *S. aureus*, with a minimum inhibitory concentration (MIC) of 75 ug/mL (seed peptides) and 100 ug/mL (pod peptides). *E. coli* and *S.* Typhi species required higher MIC, suggesting pea peptides may be less effective against these Gram negative bacteria (Rehman & Khanum, 2011). The antimicrobial activity associated with peas can reduce competition and improve detectable levels obtained during the pre-enrichment processes. However, reported activity against *Salmonella spp.* may also inhibit the rehabilitation and isolation of *Salmonella* from meat analogs containing pea protein.

In terms of nutrition, pea protein is rich in essential amino acids with high levels of lysine, however, is deficient in methionine (Gorissen et al., 2018). Pea protein differs from other plant proteins, such as soy, as it is more digestible, less allergenic, and low cost, making it desirable to both consumers and manufacturers (Lu et al., 2019).

2.3.1.2. Wheat protein

Wheat proteins such as wheat gluten are prepared by the isolation of protein from wheat flour and are commonly used in meat analog products (**Table 4**) (Day et al., 2006). Celiac disease, non-celiac gluten sensitivity, and wheat allergy are growing concerns in the U.S., with wheat being on the list of top 8 food allergens in the U.S. Despite this, wheat gluten is commonly added to meat analog products for its viscoelasticity, solubility, swelling, and nutritional content (Balakireva & Zamyatnin, 2016; Day et al., 2006; Kumar et al., 2015). While wheat protein is nutritionally beneficial, its protein quality (PDCAAS of 50% and DIASS of 45%) is not as high as that of soy (PDCAAS 86-98% and DIASS of 84-89%), as it is deficient in the essential amino acids lysine and threonine (Day et al., 2006; Mathai et al., 2017; Rizzo & Baroni, 2018).

Additionally, the protein content of wheat (7-22%) often varies due to growth conditions, with one-third of the variation being genetic (Shewry, 2009; Vogel et al., 1978). For this reason, wheat gluten is often used in combination with other plant proteins when formulating meat analogs. Samard and Ryu (2019) investigated and compared the physicochemical and texture characteristics of textured vegetable protein (TVP) made with soy and wheat gluten to beef, pork, and chicken meats. While the texture and physiochemical characteristics of the TVP did not show similar results to the meats tested, the wheat gluten extruded at 450 g/kg showed similar solubility, chewiness, and cutting strength to the poultry sample (Samard & Ryu, 2019). Similarly, Kumar, Kumar, & Sharma (2012) optimized the percentage (10%, 14%, or 18%) of wheat gluten at the cost of texturized soy protein (TSP) incorporated in meat analogs, and they concluded that 18% of wheat gluten was the optimum amount as it resulted in higher juiciness, appearance, flavor, binding ability, and overall acceptability. The mean sensory levels for the meat analogs at the different wheat gluten levels (10%, 14%, 18%) increased all sensory traits tested in meat analogs (Kumar et al., 2012)

The wheat production process contributes to contamination by pathogenic and spoilage organisms. Exposure to contaminated soil, water, insects, and animal feces can expose wheat to coliforms, *E.coli*, *Salmonella,* and other microorganisms (Bullerman & Bianchini, 2009; Myoda et al., 2019). Beyond agricultural impacts, contamination can also occur during harvesting, storage, and processing (Myoda et al., 2019). Wheat flour and wheat flour-containing products have been involved in several recalls due to the potential presence of *Salmonella* and *E. coli* (FDA, 2015c, 2019a, 2019c; PHAC, 2017; Zhang et al., 2007). When Myoda et al. (2019) screened wheat for the foodborne pathogens *Salmonella*, *L. monocytogenes,* and EHEC before storage in silos and milling, 1.23% of the wheat berries were positive for *Salmonella*, 0.44% of

the wheat berries tested positive for EHEC, and *L. monocytogenes* was not detected in any wheat berries. Not only do these findings pose a threat to public health, but the presence of bacteria could lead to competition when pre-enriching meat analog samples containing wheat gluten or wheat flour to isolate *Salmonella*. However, it is important to note that the Myoda et al. (2019) study was carried out on wheat before any processing took place. Both wheat gluten and textured wheat protein are further processed and are much less likely to be contaminated; however, if processing techniques resulted in injured pathogens, the enrichment process could serve to resuscitate the injured cells.

2.3.1.3. Potato protein

Potato is the fourth-largest crop worldwide and the second-largest protein supply grown per hectare after wheat (Bártová et al., 2015; Waglay & Karboune, 2020). The total protein content in potatoes of 4.3-4.9% dry matter is comparable to that of wheat, however, it is highly dependent on the growth region, potato variety, and year of cultivation (Lachman et al., 2005). Potato juice is a waste by-product of the potato starch industry and retains most of the proteins after the grinding of potatoes (Bártová & Bárta, 2009). Potato juice contains crude proteins, amino acids, and amides and can be utilized to filter and extract potato proteins from for use in food products (Bárta et al., 2008; Oikawa et al., 2020). However, recovering proteins that are non-denatured, soluble, emulsifying, and foaming can be an expensive process (Bárta et al., 2008). Potato proteins are advantageous for use in the food industry as they are emulsifying, foaming, gel-forming, have antioxidative activity, and have reduced allergenicity (Bártová et al., 2019; Waglay & Karboune, 2020). Potato proteins have also demonstrated antimicrobial, antifungal, and antiviral activity (Bártová et al., 2019). Bártová et al. (2019) also noted that the inhibitory protease proteins, Potide-G, AFP-J, Potamin-1, and PG-2, have antimicrobial activity against *S. aureus, L. monocytogenes, E.coli,* and *Candida albicans.* Similarly, Jin et al. (2008)

examined the effects of potato protein antimicrobial activity on pig intestinal microflora and reported inhibition of *S. aureus, Salmonella* spp., and *E. coli* growth. In the study, about 280 pigs were fed a basal diet supplemented with various potato protein concentrations from *Solanum tuberosum* L. cv. Golden valley potatoes. It was found that a minimum inhibitory concentration of 1000-1250 μg/ml potato protein was effective against all three pathogens, resulting in reduced levels of pathogens found in the feces and large intestines of the pigs. While the intestinal environmental conditions of this study differ from that of meat analogs, Jin et al. (2008) exemplifies the ability of potato proteins to act against pathogenic bacteria and as a preventative measure for pathogen contamination in pork products.

2.3.1.4. Mycoprotein

Single-celled proteins, such as mycoproteins, are protein extracts or biomass sourced from a culture of bacteria, fungi, algae, and yeast (Upadhyaya et al., 2016). The mycoproteins utilized by the meat analog company Quorn™ are produced by the continuous fermentation of the mold *Fusarium venenatum*, which has a high proportion of protein biomass and has received FDA GRAS approval (FDA, 2002; Hashempour-Baltork, Hosseini, et al., 2020; Quorn, 2020a). A pressure cycle fermenter bioreactor system produces the fungus. The cultures are maintained at a pH of 6 and 28-30 to achieve a specific growth rate (μ) of 0.17 to 0.20 h^{-1} and produce about 300-350 kg biomass h^{-1}. Following fermentation, the RNA is degraded so it can diffuse out of the cell, and the remaining biomass is heated and centrifuged to achieve the mycoprotein paste, which can be further processed to achieve the desired product texture (Dekkers et al., 2018; Wiebe, 2004). Fermentation of single-celled proteins can be completed as solid, semi-solid, submerged, or surface cultures. However, submerged fermentation can result in a greater yield. Submerged fermentation utilizes a nutrient-rich liquid substrate, with the biomass product

continuously obtained from the fermenter (Suman et al., 2015). Solid culturing is not as commonly used as it is time-consuming, difficult to automate, and has increased risk for contamination (Kim et al., 2011). Substrates used during fermentation of single-celled proteins can vary. For example, raw material substrates such as starch, molasses, fruit, and vegetable wastes can be used as well as carbohydrate substrates, which are the most widely used for single-cell protein production. Substrate choice can depend on availability, manufacturer location, and cost **(**Suman et al., 2015)

Mycoproteins are nutrient-rich meat alternatives with similar taste characteristics as animal meat. For example, the naturally high levels of sulfur-containing amino acids (methionine and cysteine) and glutamic acid in mycoproteins give it an umami flavor similar to meat (Kim et al., 2011; Trinci, 1994). Mycoprotein is composed of 45% protein and 25% fiber; it is rich in amino acids, with 41% of the total protein content being essential amino acids, making it comparable to animal protein (Finnigan et al., 2019). While mycoproteins provide similar taste characteristics to meat, consumer concerns about using a mold to produce these proteins have led to some apprehension. The misperception that mycoprotein is made from pathogenic mold has limited consumption of these products (Kim et al., 2011). However, Hasempour-Balrok et al. (2020) evaluated mycoprotein produced by submerged fermentation of *Fusarium venenatum* using date wastes and ammonium salts as substrates, and did not detect mycotoxins (zearalenone and deoxynivalenol) in the final product. Likewise, O'Donnell et al. (1998) examined Quorn™ products for the presence of trichothecene mycotoxins and did not detect any toxins in the products.

Meat analogs made from mycoproteins can lower the potential for bacterial pathogen contamination, as there is less opportunity for contamination during the production process

compared to analogs made with plant products. However, if mycoprotein is fermented with raw fruit and vegetable wastes as opposed to starch or molasses, microbiota commonly associated with the fruit and vegetable waste substrate may pose a risk. Overall, while pathogenic contamination is possible if good manufacturing practices (GMP) are neglected, the use of mycoproteins lowers this risk due to the limited processing steps and fermentation environment. There have been no reports of antimicrobial activity associated with mycoproteins, therefore these proteins would not be expected to influence the pre-enrichment process significantly.

Table 6. Microorganisms associated with plant-proteins in meat analogs and their impact on *Salmonella* pre-enrichment

Protein source	**Associated Microbiota**	**Potential impact on pre-enrichment**	**References**
Soy	Coliforms, *Klebsiella pneumoniae*	The potential presence of *Klebsiella pneumoniae* may lower pH, potentially impacting the recovery of *Salmonella* in non-buffered enrichment broth.	(Keeton & Melton, 1978; Wang et al., 2015)
Mung bean	LAB[1]	The potential presence of LAB may lower pH, potentially impacting the recovery of *Salmonella* in non-buffered enrichment broth.	(Fett & Cooke, 2003; Zheng et al., 2015)
Wheat	Coliforms, *E. coli*[2], *Salmonella*	The large potential for bacterial contamination in the food matrix could cause greater competition for nutrients, limiting the amount of *Salmonella* isolated.	(Bullerman & Bianchini, 2009; Myoda et al., 2019)
Pea	N/A	N/A	N/A
Mycoprotein	N/A	N/A	N/A
Potato	N/A	N/A	N/A

1. Lactic acid bacteria; 2.Escherichia coli

2.4. Meat analog processing

2.4.1. Extrusion

Extrusion is a common technique to manufacture meat analogs. Meat analog companies including Beyond Meat™ and Quorn™ have filed patents involving extrusion cooking (Geistlinger, 2015; Young-deok, 2007). Extrusion consists of two main techniques: low-moisture extrusion and high moisture extrusion. Low moisture extrusion is utilized to manufacture TVP by extruding plant-flours or concentrates. High moisture extrusion, typically used for meat analog production, exposes the proteins to high temperature (90-180 °C), high moisture (40-70%), high pressures (1-5 MPa), and mechanical shear stress caused by the rotation of either a single or double screw (Pietsch et al., 2017). During high moisture extrusion, 40-70% wt/wt water and 15-35% wt/wt plant material are fed into the extruder, which is then injected with 2-15% wt/wt liquid oil fat, or a combination of both (Pietsch et al., 2019; Trottet et al., 2016). During this thermomechanical process, the molecular structure of the protein changes; the protein subunits are denatured, followed by the formation of non-covalent and covalent interactions. The protein-protein interactions formed during this step result in protein aggregates. Disulfide bonds formed during this process influence the high moisture extrusion of soy protein, pea protein, and wheat gluten (Chen et al., 2011; Li et al., 2018; Pietsch et al., 2018; Pietsch et al., 2019). The extruded product then passes through the high-pressure cooling die in which the mixture cools down, and the protein forms networks depending on the flow characteristic within the die. As the product leaves the die it expands due to pressure difference and the final structure is formed (Bianchini et al., 2012; Kelley & Walker, 1999; Krintiras et al., 2015; Pietsch et al., 2018; Pietsch et al., 2019).

Meat analog texture can be altered by controlling the moisture content and cooking temperature during extrusion. Lin, Huff, & Hsieh (2000) produced soy protein isolate and wheat-

starch meat analogs using high moisture extrusion at varying moisture contents (60%, 65%, and 70%) and temperatures (137.8, 148.9, and 160 °C) and determined that a lower a moisture content resulted in a higher die pressure, a harder and chewier texture, and lower protein solubility. In contrast, at fixed moisture and high temperature, the resultant meat analog was softer and less chewy but had no significant change in protein solubility. Pietsch et al. (2019) examined the influence of high-moisture extrusion temperature (100, 125, and 155 °C), feed rate (10 and 20 kg/h), and screw speed (180, 400, 800 rpm) on the polymerization of wheat gluten and texture characteristics of the final product. Overall, thermal treatment during extrusion plays a significant role in wheat gluten polymerization. An increase in wheat gluten polymerization was correlated with the formation of fibrous, anisotropic product structure, increased hardness, and increased elasticity.

The application of high pressure, high heat, and mechanical stress during extrusion can deter the growth of potential contaminants (Anderson et al., 2017; Verma & Subbiah, 2019). *Salmonella* can survive in low moisture foods ($a_w < 0.85$), including plant-based flours. For example, in 2019, the FDA recalled Gold Mills Gold Medal Unbleached flour due to *Salmonella* contamination (FDA, 2019a). Additionally, extruded pet food, breakfast cereals, and crackers have been implicated in recalls due to *Salmonella* contamination (FDA, 2018b, 2018d, 2020c, 2020d). While extrusion conditions are unfavorable to *Salmonella* (90-180 °C, 100-10000 psi), the presence of *Salmonella* in extruded food and pet food products suggests *Salmonella* may survive the extrusion process. However, Anderson et al. (2017) examined the reduction of *Salmonella* Agona during the single-screw extrusion of oat flour at varying temperatures (65-100°C) and moisture levels (14-28%, 0.70-0.95 a_w) and concluded that processing conditions above 82°C and 0.89 a_w (typical industry conditions) resulted in a 5-log reduction of *Salmonella.*

Anderson et al. (2017) suggest that the frequent *Salmonella* contamination in extruded foods is likely due to improper processing, processing failures, and contamination post-processing. Likewise, Verma and Subbiah (2019) examined the effects of fat (5-15%), moisture content (14-26%), screw speed (75-225), and temperature (55-85°C) on *Salmonella* reduction in oat flour during double-screw extrusion. Verma and Subbiah (2019) found that a significant reduction of *Salmonella* was achieved at temperatures greater than 65°C and screw speeds between 100-150 rpm. However, as fat content increased (15%), the reduction of *Salmonella* decreased suggesting that high-fat foods may require greater temperatures, moisture content, or screw speed to achieve a 5-log reduction of *Salmonella.* While researchers have not examined the effects of extrusion processing on *Salmonella* reduction in meat analogs, industry standards indicate 95-180°C and 30-70% moisture that are typically used during meat analog processing (Geistlinger, 2015) would effectively reduce *Salmonella*. However, if processing failures occur or post-processing contamination occurs, *Salmonella* contamination of extruded foods is possible, as evident by recent outbreaks of extruded food and pet foods (FDA, 2018b, 2018d, 2020c, 2020d).

2.4.2. Texturizing hydrocolloids and protein ingredients

The addition of hydrocolloids to proteins to form fibrous products is another common practice patented by Morningstar Farms™ (Cavallini et al., 2006). Typically, 1-3 wt. % methylcellulose, is dissolved in a water and ice mixture and blended to form a cream-like substance. The hydrocolloid and vegetable plant proteins are then blended in oil, food starch, and flavoring ingredients which subsequently forms an emulsion. The emulsified mixture is then cooked to produce the meat analog product (Brackenridge et al., 2013; Cavallini et al., 2006).

2.4.3. Mycoprotein production

As previously noted in section 2.3.1.4, mycoprotein is most commonly produced by the continuous fermentation of *Fusarium venenatum* resulting in a protein-rich biomass (**Figure 1**).

After being fermented, the biomass RNA is degraded to meet safety standards using a heat shock treatment (64-65 °C). During this process, the RNA is degraded into monomers which diffuse out resulting in a loss in biomass. The remaining biomass is heated (90°C) and centrifuged to obtain a mycoprotein paste-like product with 20% solids (Wiebe, 2002, 2004). In the paste form, the mycoprotein is unordered and must be further processed to obtain a fibrous product. The mycoprotein paste is mixed with egg albumin, which acts as a binding agent, formed into a fibrous network through the alignment of the mycelia, steamed, chilled, and texturized, resulting in a product with a similar texture and structure to animal-meat (Wiebe, 2002). Texturization of the protein involves extrusion processing at a 40% moisture content, 100-10000 psi pressure, and 100-170°C. After heating at high pressure, the protein product enters the cooling die where the protein is organized (Young-deok, 2007). The extrusion process serves as the kill step in which the high pressure, high heat environment is unfavorable to pathogens, including *Salmonella* (Anderson et al., 2017).

Currently, Quorn^{TM} brands are using mycoprotein produced by *Fusarium venenatum* fungal species. However, due to increased interest in biomass protein, there has been a focus on innovations in mycoprotein production using various fungal strains. Filho et al. (2018) examined the production of vegan-mycoprotein by fermenting several edible fungal strains (*Aspergillus oryzae, Fusarium venenatum, Monascus purpureus, Neurospora intermedia,* and *Rhizopus oryzae)* in a pea-processing by-product (Ppbp) substrate. The major composition components of the Ppbp substrate used include polysaccharides (Glucans 62.4%; Galactans 2.3%), starch (56.4%), and protein (18.19%). Overall, the Ppbp substrate resulted in acceptable protein yield with and without the addition of alpha-amylase for 4 out of the five species tested, resulting in a biomass yield between about 200-300 mg per g of pea substrate, with *A. oryzae* and *N.*

intermedia resulting in fungal biomass with about 46% to 54% protein content obtained from the Ppbp substrate respectively (Filho et al., 2018).

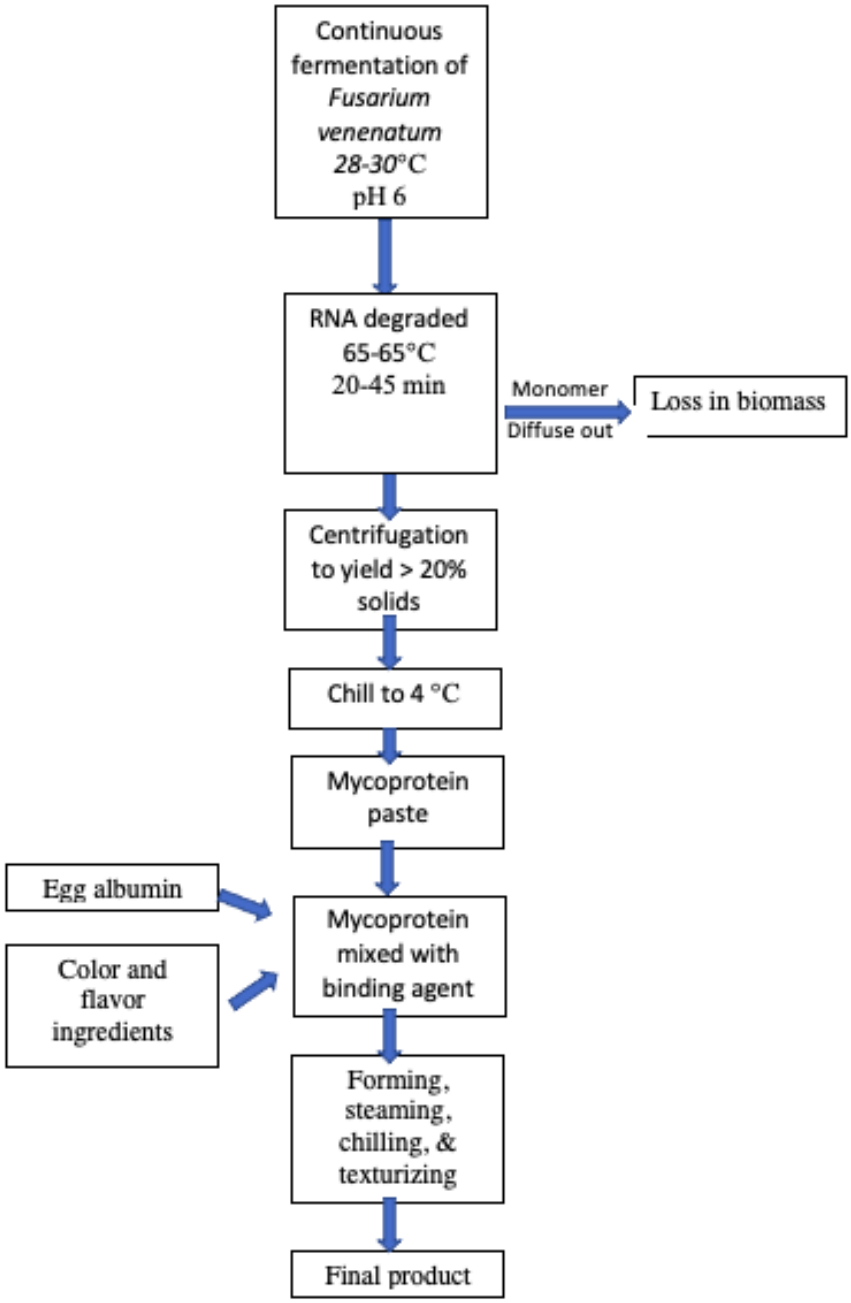

Figure 1. Unit operations involved in the production of mycoprotein as described by Wiebe (2002, 2004)

2.5. **Market available meat analog burgers**

According to the Good Food Institute (GFI), dollar sales of plant-based foods, in general, have increased 39% between 2018 and 2020, with plant-based meat retail sales rising 45% from 2019 to 2020 (Gaan, 2021). These numbers reflect the greater consumer interest in alternative meat products and the rapid growth in the industry over the last few years. The switch from animal to plant sources has seen success for plant-based milk alternatives which generated $2.5 billion in sales in 2020 (Gaan, 2021). A similar trend is now occurring for meat analogs. Plant and fungal proteins that are used to formulate meat analogs aim at satisfying the same taste, appearance, and texture as traditional animal protein food items such as beef, sausage, and chicken. Among these alternative products, there has been a focus on the replication of the typical beef or burger flavor using solely plant or fungal proteins.

In terms of the consumer base, the GFI notes that 17.6% of U.S. households purchased plant-based meat in 2020 (Gaan, 2021). The three main categories of meat analog consumers are: (1) those who consume meat analogs for health and dietary reasons (vegan, vegetarian), (2) those who believe it's better to consume plant-based products because of ethical reasons, and (3) flexitarian consumers who use meat analogs as a supplement to animal protein rather than a substitute (Grzebinski, 2020; Reinicke, 2019). Among these consumers, flexitarians make up the largest consumer base. Of the consumers who purchased plant-based burgers, 95% of them also ate meat in the same year (Settembre, 2019). This high number of "flexitarian" consumers may be attributed to the initial curiosity surrounding meat analogs. Many companies producing plant-based meat analog burgers emphasize the similarity in the amount of protein (g/serving) between the plant product and traditional animal meat products sparking consumer curiosity, which may lead to a one-time purchase of the product. Reinicke (2019) noted the continued consumer interest in plant-based meat alternatives, as 75% of consumers surveyed said they would consider

or already have bought plant-based protein, and about 40% of consumers said they would repeat the purchase, further emphasizing the growing consumer base and desire for these products. As consumers are being educated on sustainable practices and the health benefits of non-animal proteins, they incorporate these plant proteins into their diet more, leading to a growing number of "flexitarians".

According to the International Research Institute (IRI), the top five meat analog brands in terms of sales are Morningstar Farms™, Gardein™, Beyond Meat, BOCA, and Quorn™ (Grzebinski, 2020). Morningstar Farms™ holds about 41% of the market, followed by Gardein™, which holds about 14% of the market (Cheng, 2020). While these companies are the current top-selling brands in retail stores, Impossible Foods™ has also become an important competitor in the market. Impossible Foods™ is a relatively new company, founded in 2011, that, like Beyond Meat, focuses on innovating plant-based protein meat to resemble traditional burgers. In March 2020, Impossible Foods™ raised another US $500 million in funding to further their research, development, and marketing efforts. Beyond Meat went public in 2019 and had, as of 2020, a US $4 billion market value. Other well-known companies such as Tyson™, Nestle™, and Kroger™ are launching their own meat analog products. Tyson's new brand Raised & Rooted, sells vegan "meats" as well as combined plant and meat protein products such as Angus beef with pea protein (Reinicke, 2019). The Raised & Rooted burger aims to increase the nutritional value and flavor (Raised &Rooted, N.D.). Nestlé's brand Sweet Earth Foods launched the "Awesome Burger" in the United States and Switzerland which contains yellow pea protein as the major protein source in the burger (Nestle, 2019). While plant-based meat analogs are not a new market, the projected success of new and innovative companies and consumer interest has led to competition between both well-funded new market players as well as

established companies. **Table 4** summarizes the top market available meat analog products and their ingredients to exemplify the diversity of market products currently available for purchase. To better understand the competition, an analysis of the top companies including Morningstar Farms™, Gardein™, Beyond Meat, BOCA, Quorn™, and Impossible Foods™ is given in sections 2.5.1-2.5.6.

2.5.1. Morningstar Farms

The Morningstar Farms™ brand is owned by the Kellogg Company and has produced plant-based foods for about 40 years. Their plant-based products include alternative options for burgers, chicken, and sausage, all prepared in a variety of flavors (MorningStarFarms, 2020). According to IRI, Morningstar Farms™ had the greatest dollar sales of plant-based meat products in the United States in 2019, at $302.5 million. Morningstar Farm's success is due in part to being a well-established company with a wide distribution (Grzebinski, 2020). The Kellogg Company distributes Morningstar Farms™ products to most national grocery store chains, including Kroger, Albertsons, Safeway, Meijer, Prince Chopper Supermarkets, and Stater Bros (MorningStarFarms, 2020). Morningstar Farms™ has a wide range of plant-based burgers, including the "Spicy Black Bean" burger, "Veggie Lovers" burger, "Vegan Meat Lover's" burger, and the "Grillers Original" burger, all of which are sold as frozen patties. The "Vegan Meat Lovers" burger is most similar to the plant-based burgers produced by brands such as Beyond Meat and Impossible Foods™, due to claims by the brands that these meat analogs taste and feel like a traditional beef burger. Morningstar Farms™ primarily uses soy and wheat as the protein sources in its plant-based burgers. Overall, Morningstar Farm's competitive advantage includes its expansive product line, wide distribution channel, and well-established hold on the market.

2.5.2. Gardein

Gardein™, a Conagra brand, has broad distribution allowing for a large hold in the market. Their product line includes plant-based beef, chicken, pork, turkey, and fish alternatives (Gardein, 2020). In terms of plant-based burgers, Gardein™ has two frozen burger options: the "ultimate plant-based burger" and the "ultimate beefless burger". The ultimate plant-based burger is made with both pea protein and textured wheat protein. The ultimate beefless burger utilizes textured wheat protein and soy protein. Other ingredients such as beet juice are incorporated to mimic the appearance of traditional burgers (Gardein, 2020). Similar to Morningstar Farms™, Gardein™ is well established in the market and has a widely distributed product line.

2.5.3. Beyond Meat

Beyond Meat™ was founded in 2009 and has experienced rapid growth. It is currently the third top-selling plant-based meat brand, with sales growth of 135% over a 52-week period after going public in 2019 (Cheng, 2020). Beyond Meat's success and quick growth can be attributed to its innovative products such as the Beyond Burger®, which mimics the juiciness and fatty texture of a typical beef burger. The Beyond Burger® is sold like traditional burger patties in the refrigerated meat section of grocery stores. Besides being sold in retail, Beyond Burger® has generated consumer awareness by partnering with well-known foodservice companies, including Dunkin', Del Taco, and Carl's Junior (Jiang, 2019). The Beyond Burger® incorporates pea, mung bean, and rice protein, providing up to 20 g of protein. The Beyond Burger® patty has a marbling effect that mimics a traditional beef burger. These white specks are achieved using coconut oil and cocoa butter, which serve to help mimic the texture of meat. Similar to the Gardein™ burger, the Beyond Burger® also incorporates beet juice to provide the red color (BeyondMeat, 2020). These ingredients and the burger's ability to mimic the taste of

traditional beef burger gives Beyond Meat™ a competitive advantage and may explain its incredible growth. Additionally, the Beyond Burger® is soy and gluten-free, which appeals to consumers with specialty diets.

2.5.4. Boca

Boca™ is the fourth top-selling meat analog brand in terms of dollar sales and holds about 5% of the market (Grzebinski, 2020). The Boca™ brand, currently owned by the Kraft Company, has marketed its meat analog products towards the everyday consumer, not just those who consider themselves vegan or vegetarian. The brand offers various meat analog products including patties, skillet meals, bowls, falafels, chili, and nuggets (BOCA, 2019). The Boca™ All American Veggie Burger and Boca Original Vegan Veggie Burger products are both intended to emulate a traditional beef burger. The major proteins used in both products are similar to the Morningstar Farms™ products and include wheat gluten and soy protein. Due to the lack of protein diversity compared to other popular brands, and less market success compared to brands such as Morningstar Farms™ and Gardein™, Boca™ products have not successfully differentiated themselves from their competitors.

2.5.5. Quorn

Quorn™ company's meatless products include Meatless Cheesy Nuggets, Meatless Meatballs, and Meatless Salisbury Style Steaks. In terms of alternative burger products, Quorn™ offers the Meatless Gourmet Burger. Quorn™ products utilize mycoprotein as the major source of protein, along with wheat gluten, egg white, whole egg, and milk protein. The company first began innovating meatless products in England during the 1960s, when the founder recognized the inability of conventional farming to keep up with food demand due to population increases (Quorn, 2020b). Since then, Quorn™ company has made great strides improving its carbon footprint in its products. The 2018 Quorn Footprint Comparison Report reported that

mycoprotein has the smallest average water footprint compared to soy, meat, poultry, fish and was comparable to soy in the carbon and land footprints. The production of mycoprotein utilizes 90% less water and land compared to animal sources (Quorn, 2018). While there are consumer concerns regarding the production of mycoprotein due to misinformation regarding food safety, the company has seen success as consumers are becoming more aware of meat-substitute products (Kim et al., 2011). Quorn™ is the 5th top-selling meat-substitute product in the U.S. (Grzebinski, 2020). Additionally, as consumer trends shift towards plant-based proteins, recent interest in fermented protein has led to greater innovation within the meat analog market. Companies including Nature Fynd, backed by Bill Gates, and Meati are using fungi and plant protein (soy, chickpea, and fava bean) to produce plant-based breakfast patties and plant-based steak further demonstrating greater consumer acceptance of fermented protein (Wilder, 2021).

2.5.6. Impossible Foods

Impossible Foods™ produces a soy and potato protein-based product called the Impossible™ Burger that mimics the qualities of beef burgers. Similar to Beyond Meat™, Impossible Foods™ has gained brand recognition by partnering with major fast-food companies and restaurant chains, such as Burger King, Hard Rock Café, Qdoba, Red Robin, etc. (Jiang, 2019). The Impossible™ Burger can be found at restaurants and retail stores (Capritto, 2020). Since the COVID-19 pandemic, the Impossible™ Burger has become more widely available with products being sold in about 20,000 U.S. super markets, resulting in a market share increase from 5% to 55% of retail-sold plant-based patties (Shanker, 2021). Impossible™ Burgers are known for the meat flavor they produce using heme, an iron-containing compound. Genetically engineered yeast produces heme, which they incorporate into their burgers to provide flavors, colors, and aromas that mimic beef flavors. Overall, while Impossible Foods™ hasn't seen the same success in terms of dollar sales as compared to other major meat alternative companies, their increasing

brand awareness and innovative recipes give them a competitive advantage in this growing market.

2.6. Rationale and Significance

The recent appearance of diverse plant protein ingredients in meat analogs post-dates the current BAM protocol for isolating *Salmonella* from meat-analogs. The overall research goal is to compare the efficiency of three pre-enrichment broths (i.e., LB, UP broth, and BPW) in the isolation and detection of *Salmonella* in plant-based burger products containing a variety of plant protein sources. *Salmonella enterica* serotypes Agona and Enteritidis were selected for use in this study as they each have been linked to one or more ingredient in meat analogs, or identified in similar products to meat analogs (CDC, 2008; FDA, 2015a; Mohle-Boetani et al., 2009; Wierup & Häggblom, 2010). It was hypothesized that UP broth, the most nutrient-dense enrichment broth, would show the greatest repeatability of *Salmonella* detection across the various meat analogs brands tested (Hammack et al., 2001; Hammack et al., 2008; Jacobson et al., 2017; Liao & Fett, 2005). The findings of this study will help to inform the FDA and other microbial testing laboratories of the most effective pre-enrichment broth for recovering *Salmonella* from meat analogs. The research findings will ensure food safety standards are met through the accurate detection of *Salmonella* in meat analog products, ultimately ensuring consumer safety.

3. Materials and Methods

Figure 2 outlines the experimental design.

3.1. Media preparation

The following media were obtained from NEOGEN (Lansing, MI, USA) and prepared as specified by the manufacturer: LB, BPW, UP broth, Rappaport Vassiliadis (RV) broth, Tetrathione (TT) broth, Xylose-Lysine-Tegritol (XLD) agar, Hektoen Enteric (HE) agar, Bismuth Sulfite (BS) agar, Lysine Irone Agar (LIA), Tryptone Soy Agar (TSA), and Brain-Heart Infusion (BHI) broth. Triple Sugar Iron (TSI) agar was obtained from Thermo Scientific™ (Waltham, MA, U.S.A) and prepared as specified by the manufacturer. Triton X-100 was obtained from MilliporeSigma (Burlington, MA, USA) and steamed prior to used.

3.2. Meat analog products

Five meat analog burger products (MorningStar Farms® Meat Lovers Vegan Burgers, Gardein™ Ultimate Plant-Based Burger, Beyond Meat™ Beyond Burger®, Impossible Foods™ Impossible™ Burger, and Quorn™ Meatless Gourmet Burgers) were purchased from local grocery stores in Orange County, CA, and transported on ice to the laboratory. Meat analog products were stored at -80 °C.

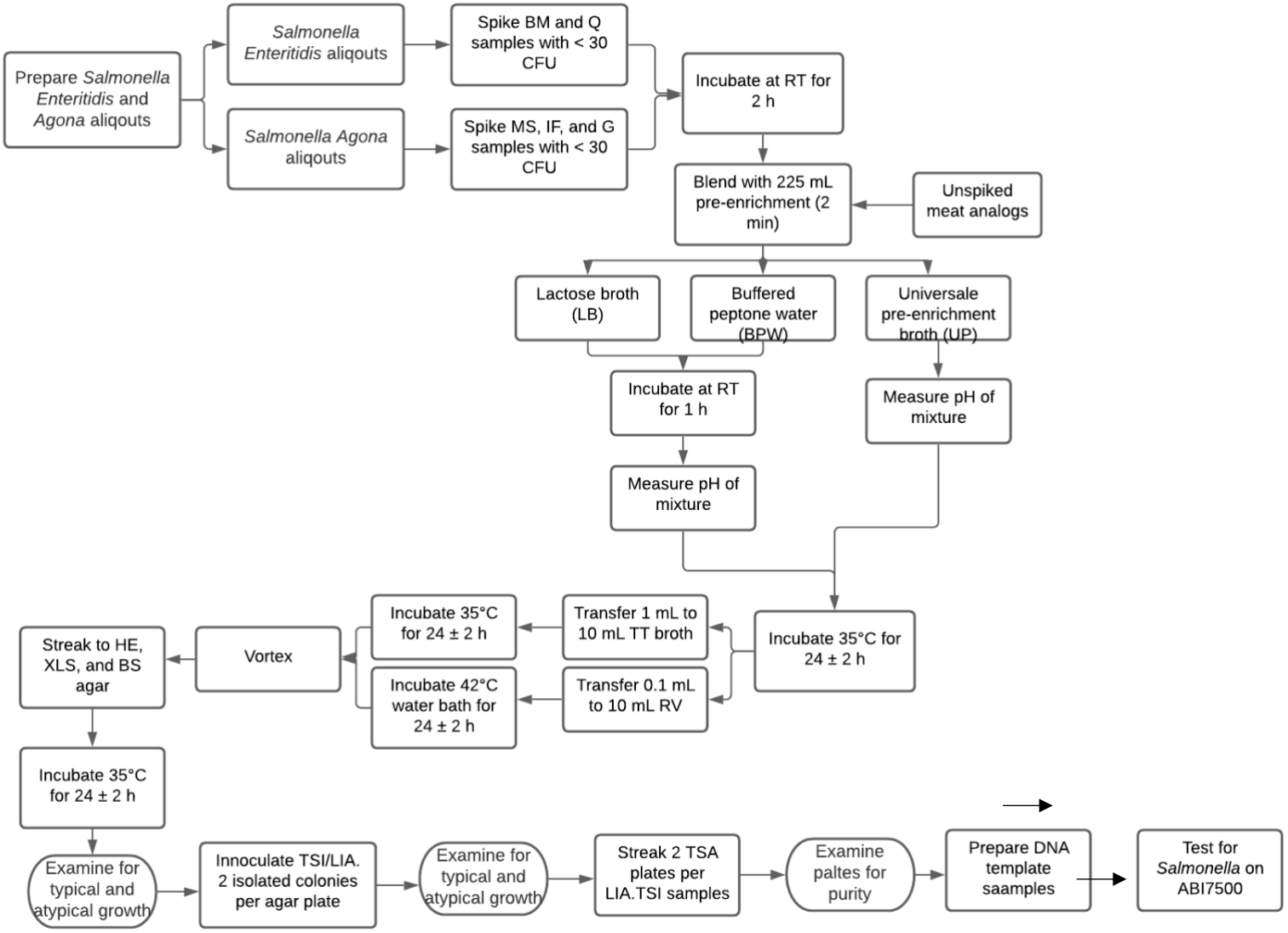

Figure 2. Schematic outline of the experimental protocol to determine pre-enrichment efficacy for the detection of *Salmonella* spp. in meat analogs

3.3. VIDAS screening

Prior to performing the experiments, the meat analogs from each brand were screened to ensure they were not previously contaminated with *Salmonella*. Samples were prepared for screening according to eBAM Chapter 5 (2020a) and screened according to AOAC Official Method of Analysis 2011.03 *Salmonella* in a Variety of Food using the VIDAS SLM assay kit (bioMérieux, Hazelwood, MO, USA). A 25 g portion of each product associated with a different lot number was aseptically removed and tested for *Salmonella*. An additional 25 g portion from the same product was inoculated with *S.* Gaminara ATCC 5695 at a level of 29 CFU/25 g product and tested to ensure that the matrix did not inhibit the screening analysis.

Each 25 g portion was pre-enriched with 225 mL sterile LB and blended for approximately 2 min. The pre-enriched samples were equilibrated at room temperature for 1 h, and then the pH was adjusted to 6.8 ± 0.2. An aliquot of approximately 1 mL of Triton-X was added to each sample, followed by incubation at 35°C for 24 ± 2 h. Next, an aliquot of 100 µL was transferred from the pre-enriched samples to SX2 broth and incubated in a thermostatically controlled circulating water bath at 42 °C for 24 h. Following incubation, an aliquot of 500 µL was transferred to a VIDAS SLM reagent strip and heated for 15 ± 1 min in a VIDAS Heat and Go block (bioMérieux). The heated VIDAS SLM reagent strip was then analyzed with the VIDAS system. Samples were only used in further testing if the un-inoculated product was negative for *Salmonella* and the inoculated product was positive for *Salmonella.*

3.4. Preparation of inoculum

Salmonella serotypes (Agona and Enteritidis) that have previously been linked to one or more of the protein sources in the sample meat analogs were selected for use in this study. Each meat analog sample was inoculated with one *Salmonella* serotype (**Figure 3**). An atypical control

culture (lactose-positive, H2S-negative *S. enterica* Diarizonae) was used to facilitate the identification of atypical colony morphology on selective agars.

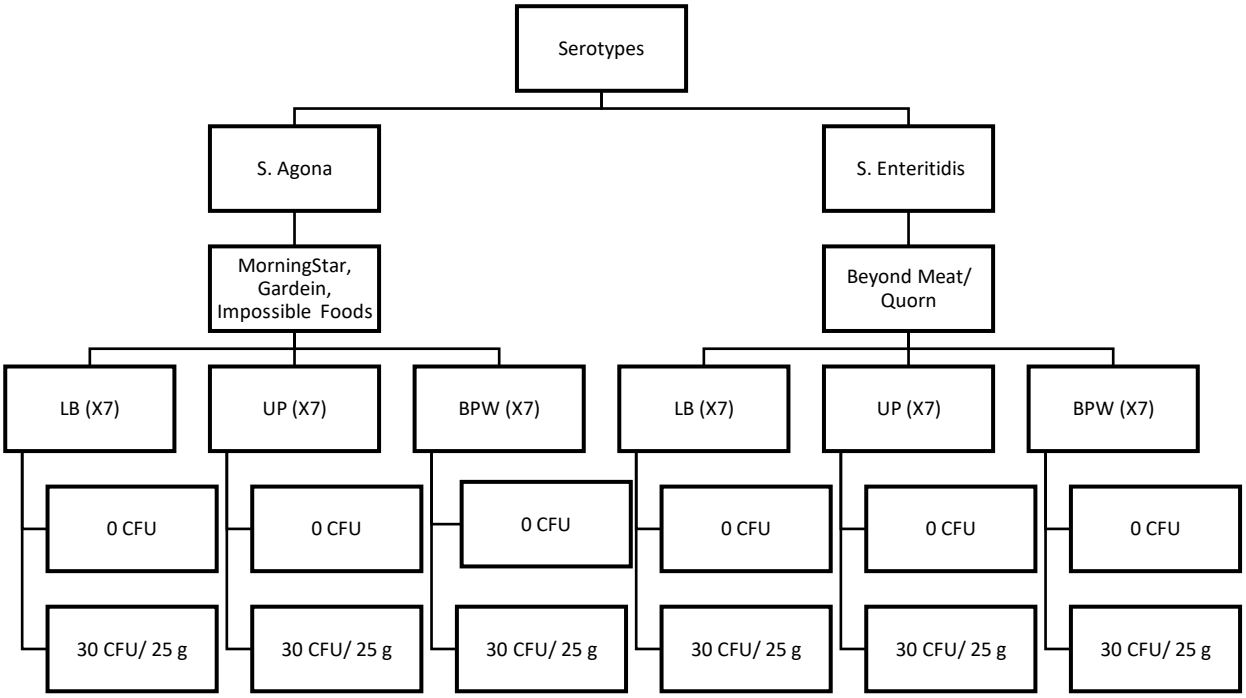

Figure 3. Meat analog spiking plan based on Salmonella serotype for pre-enrichment with lactose broth, universal pre-enrichment broth, and buffered peptone water

Environmental isolates of *S. enterica* Enteritidis (PFGE2811) and *S. enterica* Agona (FDA 2234), as well as atypical *S. enterica* Diarizonae (ATCC 29934) were obtained from the Pacific Southwest Feed and Food Laboratory (Food and Drug Administration, Irvine, CA). The *Salmonella* inoculum was prepared as described by FDA (2015b) with slight modifications. The frozen stock cultures were thawed in a 37°C water bath for a maximum of 4 min. An overnight culture was prepared for each stock culture by inoculating 10 ml of each sterile enrichment broth (LB, BPW, and UP) with a loopful of the *S. enterica* isolate and incubating for 18-20 h at 35°C. An uninoculated media control was also prepared for each enrichment broth used. Next, 10 mL of the overnight culture was transferred to 90 mL of sterile Butterfield's phosphate buffer (GVS Life Sciences, Sanford, ME, USA) to produce a 10^{-1} dilution. Successive serial dilutions were

performed by transferring 10 ml of the previous dilution to 90 ml of sterile Butterfield's phosphate buffer until the desired dilution of 10^{-6} was obtained. A portion (50 ml) of the 10^{-6} dilution was replaced with 30 mL of sterile phosphate buffer and 20 mL sterile glycerol to produce a 20% glycerol solution. The solution was mixed by shaking 25 times in a 30 cm arc for approximately 7 seconds and 1 mL aliquots of the 10^{-6} diluted sample were transferred to microcentrifuge tubes. The aliquots were then stored at -80°C for up to six months.

Cell concentrations were determined using TSA plates. After storing the aliquots at -80°C overnight, three 1 mL aliquots for each pre-enrichment broth were thawed in a 37°C water bath for no more than 4 min. The aliquots were enumerated by spread plating a total of 0.1 mL to 2 TSA plates (6 plates per 3 aliquots). The TSA plates were incubated overnight at 35 °C and the primary counts were recorded. Based on these values, the aliquot volume was adjusted so that the inoculum yields an average of 30 CFU or less.

3.5. Inoculation

FDA (2019b) Matrix Extension Verification Procedures 5.1.1 were followed for the comparison of LB, BPW and UP broth. For all pre-enrichments, seven replicates of each of the five selected meat analog products were inoculated with ≤30 CFU *Salmonella enterica* per 25 g of product and an additional seven un-inoculated replicates were tested as controls for process contamination (**Figure 3**Meat analog products were stored at -80 °C.). The Beyond Meat™ Beyond Burger® and Quorn™ Meatless Gourmet Burgers were spiked with *S.* Enteritidis due to previous related outbreaks with mung bean and egg white. The remaining meat analog burgers (i.e., MorningStar Farms® Meat Lovers Vegan Burger, Gardein™ Ultimate Plant-Based Burger, and Impossible Foods™ Impossible™ Burger) were spiked with *S.* Agona due to previous related outbreaks with wheat and soy containing products. Sample inoculation was carried out as described by Rosen et al. (2020). Frozen samples were placed in a 4 °C incubator overnight to

thaw. A 25-g portion of each thawed meat analog sample was weighed out using an analytical balance (Mettler Toledo, Chino, CA, USA) and placed in a sterile blending container. Each sample was spot-inoculated with ≤30 CFU of the *Salmonella* suspension. The inoculum was dispersed in at least three different locations of the sample. Inoculated samples were then incubated in a biosafety cabinet at room temperature for 2 h to allow for absorption of *Salmonella.*

3.6. Pre-enrichment of meat analog samples

Samples enriched with lactose broth were prepared as described by eBAM Chapter 5 subsection C, part 15 (Meat, meat-substitutes, etc.). Inoculated samples (25 g) were blended with 225 ml of sterile LB for 2 min. The mixture was then held at room temperature for 60 ± 5 min. The samples were swirled to mix, and the pH was determined and adjusted to 6.8 ± 0.2 if necessary. Up to 2.25 ml of steamed Triton X-100 was added to each sample or until foaming occurs. Samples with loosened jar caps (1/4 turn) were then incubated for 24 ± 2 h at 35± 2 °C.

Samples enriched with universal pre-enrichment (UP) broth were prepared as described by eBAM Chapter 5 subsection C, part 27 (Fresh leafy green vegetables, herbs, and sprouts) with slight modifications. Inoculated samples (25 g) were blended with 225 ml of UP broth for 2 min. The mixture was incubated with loosened jar caps (1/4 turn) for 24 ± 2 h at 35 ± 2 °C.

Samples enriched with buffered peptone water (BPW) were prepared as described by eBAM Chapter 5 subsection C, part 28 (Animal food) with slight modifications. The inoculated 25 g samples were blended with 225 ml of BPW broth for 2 min. The homogenized mixture was incubated at room temperature for 60 ± 5 min. The samples were swirled to mix and the pH of each sample was then determined and adjusted to 6.8 ± 0.2 if necessary. Samples were incubated with loosened jar caps (1/4 turn) at 35 ± 2 °C for 24 ± 2 h.

3.7. Isolation of *Salmonella*

Salmonella isolation was conducted as described by eBAM Chapter 5 subsection D. Following incubation, samples were gently shaken to disperse contents. Using aseptic technique, 0.1 ml of the sample mixture was transferred to 10 mL Rappaport-Vassiliadis (RV) medium and 1 ml of the mixture was transferred to tetrathionate (TT) broth. The samples were then vortexed and incubated. Samples in RV medium were incubated for 24 ± 2 h in a water bath set at 42 ± 0.2°C water bath and TT samples were incubated for 24 ± 2 h in an incubator set at 35 ± 0.2°C.

Following incubation, the samples were vortexed, and a 3 mm inoculating loop (10 µl) was used to streak the RV and TT broth samples onto BS, XLD and, HE agar. The streak plates were incubated for 24 ± 2 h at 35°C. Following incubation, plates were examined for typical and atypical colonies as described in **Table 7**. Upon examination, typical *Salmonella* colonies were selected from each of the agar plates (total of 6 colonies per sample) for confirmation with TSI and LIA slants. The slants were incubated at 35°C for 24 ± 2 h with the caps loosely secured to maintain aerobic conditions and prevent excessive H_2S production. One LIA and one TSI slant per sample that presented typical growth, or atypical growth if no typical samples were present, were streaked to 2 TSA plates (**Table 7**). The TSA plates were incubated at 35°C for 24 ± 2 h, followed by confirmation with real-time PCR.

Table 7. Typical and atypical *Salmonella* colony morphology on BS, XLD, and HE agar

Agar	Typical *Salmonella* colony morphology	Atypical *Salmonella* colony morphology
Bismuth sulfite (BS) agar	Brown, gray or black colonies. Can have a metallic sheen. As incubation time increases surrounding media turns black and may see a halo effect	Green colonies, surrounding medium may darken a little
Xylose lysine deoxycholate (XLD) agar	Pink colonies. Some colonies may have large, black, glossy centers and others can be completely black, or have no black center	Yellow colonies with/without black centers
Hektoen enteric (HE) agar	Blue-green and blue colonies with black centers. Black centers may be large, and colony can appear completely black	Yellow colonies with/without black centers
TSI	Alkaline (red) slant and acid (yellow) butt with or without H2S production (black)	Alkaline (red) slant and alkaline (red) butt; Acid (yellow) slant and acid (yellow butt)
LIA	Alkaline slant (purple) and alkaline butt (purple) and can produce H2S (black)	Acid (yellow) and acid butt (yellow) Alkaline slant (purple) and acid butt (purple)

FDA (2020a). Bacteriological Analytical Manual, Chapter 5 *Salmonella*. (2020), from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-5-*Salmonella*

3.8. Confirmation using real-time PCR

Typical *Salmonella* colonies were confirmed with real-time PCR using the ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). *Salmonella* confirmation was carried out as described by BAM Chapter 5 subsection E, part 9c (Real-time PCR confirmation test). To prepare samples for PCR testing, one colony from each TSA plate (2 colonies per sample) was selected and grown in an overnight culture of BHI broth. Each time a DNA template was prepared, positive and negative extraction controls were prepared with the same BHI broth. The DNA templates were prepared by transferring 250 µl overnight culture in BHI broth to a 1.5 mL microcentrifuge tube. The samples were then centrifuged for 5 min at 10,000 g, the resulting supernatant was removed, and the pellet was resuspended in 250 µl sterile water. The samples were then heated at 100°C for 20 min and immediately cooled on ice for 5

min. Next, the samples were centrifuged for 5 min at 10,000 rpm. The supernatant was then removed and saved as the DNA template. The DNA template was stored frozen (-80°C) for future PCR testing (**Figure 4**).

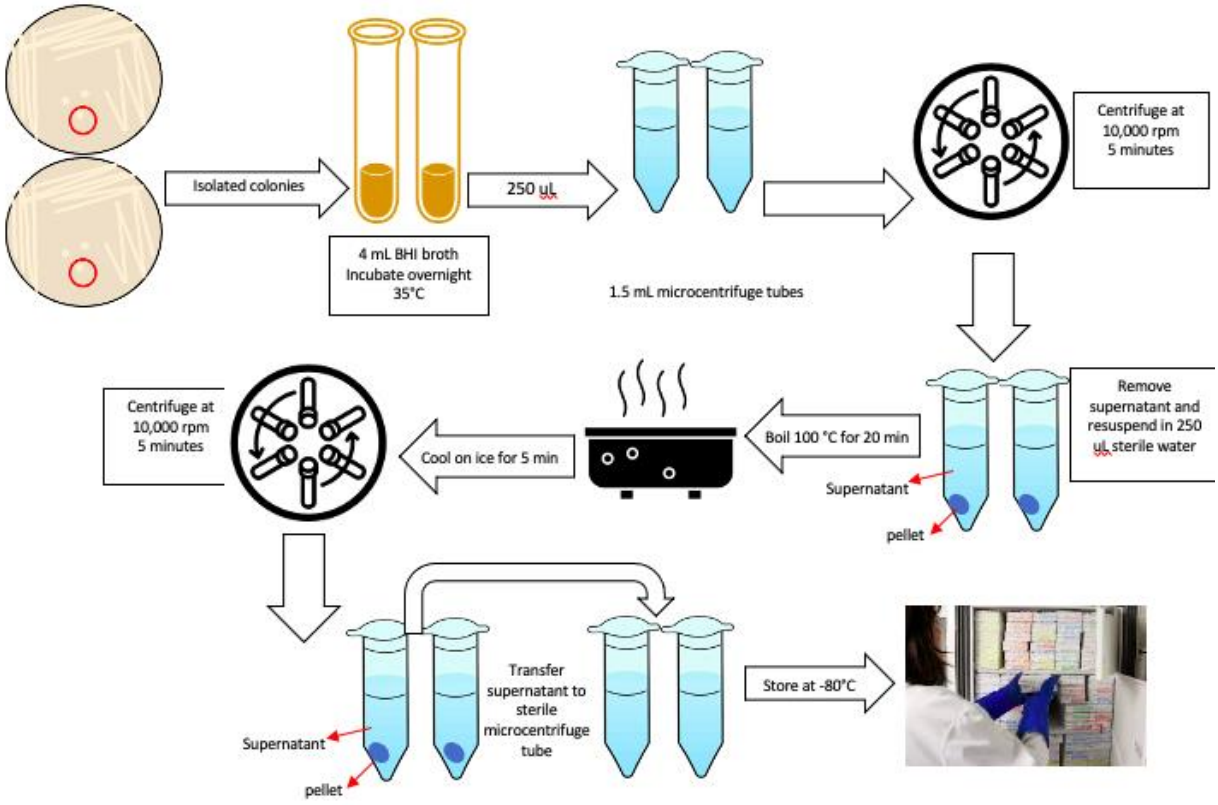

Figure 4. Preparation of DNA template samples tested using real-time PCR

Preparation of the real-time PCR master mix and sample components was performed as described in BAM, Chapter 5 subsection E, part 9c. The master mix components (**Table 8**) were combined in a 1.5 mL microcentrifuge tube, vortexed, and briefly centrifuged. The master mix was diluted 2.5X and a QC run was performed on the ABI 7500 Fast instrument. The QC run was completed for each new batch created and involved testing the master mix batch with three no template controls and three positive template controls on the ABI 7500 instrument. The real-time PCR master mix was then stored in the dark at 4°C until ready for use.

To assemble the reaction components, 8.0 µl/reaction of 2.5X working solution of the real-time PCR Master Mix, 10.0 µl/reaction of TaqMan Fast Advance Master Mix, and 2.0 µl/reaction of the DNA template (sample or control) were combined. The samples were then run on the ABI 7500 Fast instrument and examined for primary fluorescent curves that crossed the threshold value, indicating *Salmonella* positive results (FDA, 2020).

Table 8. Components for 1 tube real-time PCR of real-time PCR Salmonella Master Mix (10X) as described in BAM Chapter 5.

Component	**Volume (µl)**
Primer *Sal* 1598 (10 µM solution)	16.0
Primer *Sal* 1859 (10 µM solution)	16.0
Primer IAC F (10 µM solution)	6.7
Primer IAC R (10 µM solution)	6.7
Probe *Sal* 1631PFAM (10 µM solution)	20.0
Probe IAC30PCy5 (10 µM solution)	25.0
20% sucrose (0.22 µM filter-sterilized)	1.3
IAC plasmid DNA template (0.075 pg/ µl)	2.5
PCR grade water	5.8
Total	100

FDA. (2020). Bacteriological Analytical Manual, Chapter 5 *Salmonella*. (2020), from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-5-*Salmonella*

3.9. Statistical analysis

The pre-enrichment broths were statistically compared based on their ability to allow for positive real-time PCR detection of *Salmonella* in the inoculated samples and no detection in the un-inoculated samples (i.e., no false positives and no false negatives). The number of positive real-time PCR results for *Salmonella* using each pre-enrichment broth was compared across all products and within each product using the Test for Equality of Proportion (p-value < 0.05) in R Studio version 1.1.463 (2018) (RStudio, Vienna, Austria). The Kruskal Wallace H test followed by the Dunn test was to determine if there was a significant difference in the number of LIA and TSI slants with atypical growth for each pre-enrichment broth compared across all products and within each product tested.

4. Results and Discussion

4.1. VIDAS Screening

Pre-screening with VIDAS showed that none of the product lots used in this study were contaminated with *Salmonella* and all laboratory controls gave the expected results. The negative VIDAS results ensured that the *Salmonella* detected in the products resulted from the inoculation procedure and not from a contaminated product. Furthermore, all inoculated samples subjected to pre-screening were positive with VIDAS, indicating that the matrix did not adversely influence the screening assay.

4.2. Comparison of pre-enrichment broths for the detection of *Salmonella* in meat analog products

All uninoculated samples tested on LB, BPW, and UP obtained negative real-time PCR results for *Salmonella*, indicating that no instances of processing contamination occurred. When comparing the real-time PCR results across all five meat analog products (**Table 9)**, the greatest proportion of positive *Salmonella* detections was found with LB, which had a positive detection rate of 67/70 (95.7%). In comparison, BPW and UP broth each showed a slightly lower detection rate of 62/70 (88.6%). However, there was no significant difference (p-value > 0.05) in the number of positive *Salmonella* results obtained from each pre-enrichment broth when comparing the combined results for all meat analog products. These findings suggest that it is equally likely to detect *Salmonella* in the meat analog products tested using BPW, LB, or UP broth for pre-enrichment.

When the three pre-enrichment broths were compared for each product separately, there were no significant differences among the broths, with the exception of the Beyond Meat™ burgers (**Table 9).** For the Beyond Meat™ burgers, the number of positive *Salmonella* detections with

real-time PCR was significantly ($p < 0.05$) greater for LB (13/14, 92.9%) as compared to UP broth (7/14, 50%). Samples enriched in BPW showed a detection rate of 9/14 (64.3%), which was not significantly different from LB or UP broth. Interestingly, the Beyond Meat™ product showed the lowest detection rates overall, with 29 out of 42 detections (69%) for the three broths combined.

All inoculated Quorn™ and Morningstar Farms™ products tested positive for *Salmonella* when enriched with all pre-enrichment broths tested (100% detection rate). The inoculated Gardein™ product also showed a high detection rate, with 41 out of 42 (97.6%) positive *Salmonella* detections. LB and UP broth both showed 100% detection for this product, while BPW had one false negative (92.9% detection rate). The inoculated Impossible Foods™ product showed the highest detection rate (100%) with UP broth and a detection rate of 86% for BPW and LB. Despite the varying detection rates for the Gardein™ and Impossible Food™ products, there were no significant differences in the number of positive test samples (p-value > 0.05) when comparing the three pre-enrichment broths for these samples. Overall, LB broth consistently showed detection rates of 85.7-100% across the five products tested. In comparison, BPW and UP broth showed similar performance for 4 of the 5 products, but had lower detection rates for the Beyond Meat™ product.

Table 9. Positive real-time PCR results for *Salmonella* for the different pre-enrichment broths and inoculated meat analog products tested in this study.

Meat analog products	***Salmonella enterica* serotype**	**Major protein ingredients**	**Number of positive *Salmonella* real-time PCR results/total number of replicates**		
			BPW[1]	**LB[2]**	**UP[3]**
Gardein™ Ultimate Plant-Based Burger	S. Enteritidis	Textured pea protein, textured wheat protein, vital wheat gluten	14/14 [a]	12/14 [a]	14/14 [a]
Impossible Foods™ Impossible™ Burger	S. Enteritidis	Soy protein concentrate, potato protein, soy protein isolate	12/14 [a]	12/14 [a]	13/14 [a]
MorningStar Farms® Meat Lovers Vegan Burgers	S. Enteritidis	Wheat gluten, soy protein isolate, soy flour	14/14 [a]	14/14 [a]	14/14 [a]
Beyond Meat™ Beyond Burger®,	S. Agona	Pea protein, rice protein, mung bean protein	9/14 [ab]	13/14 [a]	7/14 [b]
Quorn™ Meatless Gourmet Burgers	S. Agona	Mycoprotein, egg white, wheat flour, milk protein concentrate.	14/14 [a]	14/14 [a]	14/14 [a]
Combined results	N/A	N/A	63/70 [a]	65/70 [a]	62/70 [a]

[1] Buffered peptone water
[2] Lactose broth
[3] Universal pre-enrichment broth
[ab] Different superscript values in the same row indicate a significant difference (p-value < 0.05) according to the Test for Equality of Proportions

Although the real-time PCR results were the main determinant of pre-enrichment broth performance, the results of LIA and TSI slants were also compared. For each sample, 6 LIA slants and 6 TSI slants were processed and examined for typical and atypical growth. Alkaline slants with or without H2S production in the butt were classified as 'typical' for LIA or TSI. There were no significant differences (p-value > 0.05) in the number of typical LIA or TSI slants observed with each of the three broths when comparing the combined results for all meat analog products. However, when comparing the results for individual meat analog products, a significant difference (p-value < 0.05) was identified for the inoculated Beyond Meat™ samples, in which the number of slants for samples enriched in LB with typical LIA growth (37/42) was significantly greater than that for samples enriched in UP broth (23/42) and BPW (25/42). These results support the findings from real-time PCR, in which LB performed equally well or better than BPW and UP broth for pre-enrichment of meat analog samples.

Table 10. Number of LIA and TSI slants replicates demonstrating typical growth per brand and pre-enrichment broth type.

Meat analog products	***Salmonella enterica* serotype**	**Number of replicates with typical *Salmonella* growth on LIA slants/total number of replicates**			**Number of replicates with typical *Salmonella* growth on TSI slants/total number of replicates**		
		BPW[1]	**LB[2]**	**UP[3]**	**BPW**	**LB**	**UP**
Gardein™ Ultimate Plant-Based Burger	*S.* Enteritidis	41/42[a]	41/42[a]	42/42[a]	40/42[a]	39/42[a]	42/42[a]
Impossible Foods™ Impossible™ Burger	*S.* Enteritidis	33/42[a]	39/42[a]	41/42[a]	40/42[a]	38/42[a]	41/42[a]
MorningStar Farms® Meat Lovers Vegan Burgers	*S.* Enteritidis	42/42[a]	42/42[a]	42/42[a]	42/42[a]	42/42[a]	42/42[a]
Beyond Meat™ Beyond Burger®,	*S.* Agona	25/42[a]	37/42[b]	23/42[a]	29/42[a]	25/42[a]	22/42[a]
Quorn™ Meatless Gourmet Burgers	*S.* Agona	40/42[a]	39/42[a]	42/42[a]	35/42[a]	32/35[a]	35/42[a]
Combined results	N/A	182/210	198/210	193/210	186/210	176/210	182/210

[1] Buffered peptone water
[2] Lactose broth
[3] Universal pre-enrichment broth
[ab] Different superscript values in the same row for the same medium (i.e., LIA or TSI) indicate a significant difference (p-value < 0.05) according to the Kruskal Wallace H test and Dunn test

4.3. Potential influence of background microbiota on pre-enrichment efficacy

Pre-enrichment efficacy is determined by the medium's ability to repeatedly allow for the isolation of *Salmonella* at low levels (< 30 CFU) (FDA, 2019b; Jacobson et al., 2017). Factors that influence the efficacy of pre-enrichment media and recovery of *Salmonella* include pre-enrichment composition, the presence of competitive bacteria, the extent of *Salmonella* contamination, the extent of stress or injury to the cells, and the biological and chemical characteristics of the food matrix (Daquigan et al., 2016; Jacobson et al., 2017). All samples, except the MorningStar Farms® Meat Lovers Vegan Burgers, contained background microflora, as evident by atypical growth on the uninoculated negative control samples.

The presence of background microbiota may have been due to the survival of native microorganisms in raw ingredients or post-processing contamination of meat analog products. While the extrusion process would be expected to inactivate human pathogens or significantly reduce the bacterial load in the food item, the final product is not considered sterile based on a 12-log reduction standard (Bianchlni et al., 2012; Kelley & Walker, 1999). Additionally, many of the market available meat analog burgers are processed by mixing the extruded plant protein with other ingredients, including fats, hydrocolloids, vitamins, minerals, flavors, and colorants, to form the final burger product (Lupo, 2019). These ingredients do not go through the extrusion process and may contribute to contamination of the product. Organisms may also be introduced to the product before packaging, especially if the manufacturing environment is not sterile (Anderson et al., 2017; Singh et al., 2007). The presence of background microbiota in 4 out of the 5 brands tested may have led to nutrient competition during pre-enrichment and contributed to false-negative results. In support of this theory, MorningStar Farms® Meat Lovers Vegan Burgers, the only product that did not have any growth of background microbiota, showed 100%

positive *Salmonella* detections in all three pre-enrichment broths tested (**Table 9**). The lack of competition from background microbiota likely allowed *Salmonella* to proliferate equally well in all three pre-enrichment broths. On the other hand, the Quorn samples also showed 100% detection with all three pre-enrichment broths despite the presence of background microbiota. These results suggest that additional factors, such as the diversity of the microbiota or the presence of specific ingredients in the product, may also influence the efficacy of the pre-enrichment broth.

The lower *Salmonella* detection rates observed with the Beyond Burger® meat analogs may also be attributed to increased competition during pre-enrichment due to background microbiota. Pre-enrichment media is non-selective; therefore, all organisms present in the food sample have the potential to grow in these favorable environments (Budu-Amoako et al., 1992; Chen et al., 2019). UP broth has the greatest number of added nutrients (magnesium sulfate, ferric ammonium citrate, sodium pyruvate, tryptone, peptones, dextrose, and phosphate buffers). While successful for low acid food environments, these added nutrients may have supported the growth of other microbes present in the product leading to greater competition for *Salmonella* proliferation, resulting in a significant number of false-negative detections (FDA, 2020a; Hammack et al., 2001; Jacobson et al., 2017; Liao & Fett, 2003; Wang et al., 2015; Zheng et al., 2015). A similar trend was seen with BPW. The added nutrients and the balanced pH due to the presence of phosphate buffers resulted in a more favorable environment for microbial proliferation, leading to greater competition for nutrients.

In contrast, the greater detection rate found with Beyond Meat^{TM} products enriched in LB is likely due to a combination of the background microflora present in the product and the lack of phosphate buffers in this enrichment broth. The absence of phosphate buffers may have served as

a selective agent in LB, as *Salmonella* can out-survive organisms at a lower pH (Jacobson et al., 2017). Meat analogs have a relatively neutral pH of ~6.2. If lactose fermenters were present in small amounts, the production of lactic acid may have resulted in a decreased pH making the pre-enrichment environment less favorable to native microbes but still tolerable for *Salmonella* (Gómez et al., 2019; Jacobson et al., 2017). This would have led to decreased competition for pre-enrichment nutrients allowing *Salmonella* to better proliferate in LB, resulting in a greater number of positive detections.

TSA agar plates were utilized to assess samples for purity and isolate colonies before real-time PCR testing. Growth of the spiked samples on the TSA plates typically appeared as creamy, white, opaque circular colonies. However, all Beyond Meat™ samples enriched in UP and BPW that resulted in at least one false-negative result for *Salmonella* had atypical growth, with yellow-tan, translucent spread out colonies, and a yeast-like aroma (**Figure 5**). Likewise, several of the TSI and LIA slants associated with these samples showed atypical results, indicating the presence of an organism other than H2S-positive *Salmonella* (**Table 7**). On the other hand, while Beyond Meat™ samples enriched in LB showed typical and atypical S*almonella* growth on the TSI and LIA slants, all samples appeared typical on TSA plates.

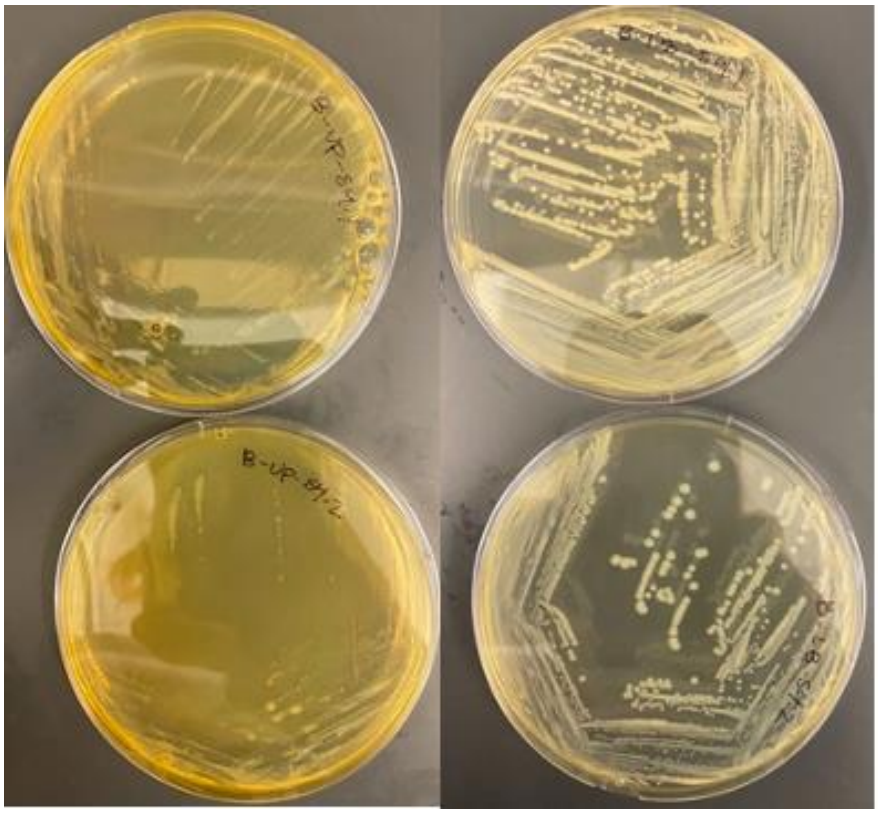

Figure 5. TSA streak plates of an inoculated Beyond Burger sample enriched in UP that tested negative for Salmonella (left) and uninoculated Beyond Burger enriched in LB that tested positive for Salmonella (right). Both meat analog samples were obtained from the same package within 4 days of each other

It is possible that yeast, lactic acid bacteria (LAB), and/or other spoilage organisms out-competed *Salmonella* during pre-enrichment, leading to false-negative results. Mung bean, pea, and rice are the major plant proteins in the Beyond Burger® samples tested. Zheng et al. (2015) reported psychotrophs, LAB, *pseudomonas spp.*, yeast and molds, and coliforms as background microflora in mung bean sprouts. Oliveira et al. (2012) reported bacterial counts of 4.9 X 10^4 and 6.2 X 10^4 CFU/g over a 90-day storage period of rice bran samples treated by milling and extrusion. Youssef et al. (2020) reported that the fermentation of pea protein with co-culture of LAB and yeast can improve sensory characteristics of pea protein and reported the biomass concentration of 4.2-6.0 X 10^8 CFU/mL bacteria and 0.7-4.4 X 10^8 CFU/mL yeast after fermentation of the plant protein. Zokaityt et al. (2021) examined the impact of combined

fermentation and extrusion process on wheat bran and reported microbial counts between 3-8.5 log CFU/g when fermented with *Lactobacillus paracasei* and extruded at a temperature between 115-130°C and screw speeds between 16-25 rpm. Under the same parameters, Zokaityt et al. (2021) reported yeast and mold growth between 4-4.5 log CFU/g. These findings demonstrate the survivability of LAB and yeasts during extrusion.

Overall, while the exact nature of the background microbiota in these meat analogs is unknown, existing microbes likely led to competition for nutrients during pre-enrichment, resulting in reduced growth of *Salmonella* for some of the products. Future studies identifying native and spoilage microorganisms in various meat analogs brands would elucidate competitive microbes in these products.

4.4. Potential influence of antimicrobial activity on pre-enrichment efficacy

It was hypothesized that meat analogs containing soy, pea, and potato protein would require a more nutrient-rich pre-enrichment broth (i.e. UP broth) due to previous reports of these proteins' antimicrobial activity against *Salmonella spp.* (Bártová et al., 2019; Dhayakaran et al., 2015; Jin et al., 2008; Pina-Perez & Perez, 2018; Rehman & Khanum, 2011; Sitohy et al., 2013). This hypothesis was not supported, as no significant difference (p-value > 0.05) was identified between the number of *Salmonella*-positive detections for meat analogs brands containing soy and potato protein (Impossible Foods™, MorningStar Farms™) when enriched with UP, LB or BPW (**Table 9**). While a significant difference was observed in Beyond Meat™ samples, which contain pea protein, when enriched with UP broth versus LB; Gardein™ samples, which also contain pea protein, showed no significant difference in the number of *Salmonella* positive detections across all three pre-enrichments. These findings suggest pea protein did not play a role in the effectiveness of the broth. Likewise, UP broth, which is more nutrient-rich, was less

effective in pre-enriching Beyond Meat™ samples than LB, suggesting that differences in effectiveness between broths may be attributed to increased nutrient competition during pre-enrichment rather than antimicrobial activity.

4.5. Cost considerations

As no significant difference was identified in the frequency of *Salmonella*-positive results obtained when the three pre-enrichment broths were compared across all meat analogs, other factors, including cost, may be considered. **Table 11** breaks down the cost of pre-enrichment per 25-g sample of meat analog. While BPW has the highest price (US$100.80) per 500 g package, less BPW (g) is required to produce 225 mL of the pre-enrichment. LB results in the highest cost per sample (US$2.91) due to the use of Triton X-100. Tergitol Anionic 7 may be used as a less expensive alternative to Triton X-100, with a cost savings of $1.10/sample (based on pricing from Fisher Scientific). However, the cost of processing a 25-g sample with LB broth and Tergitol Anionic 7 (US$1.81) is still greater than the cost associated with UP (US$1.36/sample) or BPW (US$0.76/sample). While it is recommended that LB be used for the Beyond Meat™ product, BPW or UP broth may be more economically feasible when testing the other meat analog products in this study. In order to reduce costs associated with the use of LB, future research is needed to verify whether a surfactant, such as Triton X-100 or Tergitol Anionic 7, are necessary when testing meat analogs.

Table 11: Cost breakdown of each pre-enrichment broth tested (LB, BPW, and UP) per 25 g meat analog sample

	Pre-enrichment with lactose broth	Pre-enrichment with buffered peptone water	Pre-enrichment with universal pre-enrichment broth
Cost per 500 g container of media	$84.00	$100.80	$79.90
Cost of additional reagents for use with 500 g media	$107.10 per 100 mL Triton X-100	N/A	N/A
Grams of pre-enrichment media per sample	2.93 g LB per 225 mL	4.5 g BPW per 225 mL	8.55 g UP per 225 mL
Grams of additional media per sample	2.25 mLTriton X-100 per 225 mL	N/A	N/A
Cost per sample	**$2.91** ($0.50 per 225 mL LB + $2.41 per 2.25 mL Triton X-100)	**$0.76** per 225 mL BPW	**$1.36** per 225 mL UP

Pricing obtained from Fischer Scientific website and reported in USD. All LB (Catalog No. 50-201-5168), BPW (Catalog No. 50-201-5162), and UP (Catalog No. 50-2-105279) prices are for NEOGEN manufactured products. Triton X-100 listed price is for products manufactured by Millipore Sigma (Catalog No. M1122980101**)**

5. Conclusion

Overall, the results of this study reveal that LB, BPW, and UP broth are equally effective in allowing for detection of *Salmonella spp.* in meat analogs formulated with various protein ingredients. While the greatest *Salmonella* detection rates overall (95.7%) were observed with LB as compared to BPW and UP broth (88.6% for both), the results were not statistically significant. The one exception to this was in the case of the Beyond Meat™ product, which showed significantly greater detection rates when pre-enriched with LB as compared to UP broth. Instances of false-negative detections was likely due to the presence of background microbiota, resulting in increased competition for nutrients during pre-enrichment and difficulty in isolating *Salmonella*. These results suggest that LB, the current BAM pre-enrichment broth, is the most effective at enriching and detecting *Salmonella* from the Beyond Meat™ product. However, when selecting an appropriate pre-enrichment media to detect *Salmonella* in other

meat analog products, factors including cost of materials and broth availability may be additional deciding factors. Specifically, BPW and UP broth are both more cost-effective than LB, and showed strong performance in 4 of the 5 meat analog brands tested.

6. Recommendations for future research

It is recommended that future research be conducted with a wider sample size and selection. The meat analog market continues to grow as more consumers begin to accept these products and change their dietary habits (GVR, 2020). This study focused on only 5 brands of meat analogs out of the current 21 major brands in 2021 which mimic the qualities of beef burgers. However, available market products include meat analogs that mimic the taste, sensory, and nutrition qualities of pork sausages, chicken, turkey, and other popular meat products (MeticulousMarketResearch, 2020). Sampling a more comprehensive array of meat analog products and a wider variety in brands may reveal differences in pre-enrichment efficacy depending on the biological and chemical composition of the food matrix.

Additionally, when completing this study atypical and non-*Salmonella* growth was seen across most brands. Completing a similar study to verify the BAM protocols for other pathogens of concern in meat analog products may benefit the safety of the products and the community. Likewise, identifying the background microbiota associated with meat analogs may reveal the extent of competition for nutrients during pre-enrichment of these products.

Lastly, a future study analyzing the impact of Triton X-100 on pre-enrichment efficacy is warranted. The FDA BAM currently requires the use of Triton X-100 for pre-enrichment of meat analogs in LB. Surfactants like Triton X-100 aid in the breakdown of lipids during the enrichment process (D'Aoust et al., 1982). However, D'Aoust et al. (1982) saw no difference in the frequency of *Salmonella* detections when using Triton x-100, tergitol-7, and several other

surfactants during the enrichment of high fat food and animal feed in nutrient broth supplemented with 3% surfactant. Furthermore, Triton X-100 adds considerable cost to the pre-enrichment procedure. Therefore, a study into whether Triton X-100 is necessary for use with LB when testing meat analogs should be conducted.

7. References

Alekel, D. L., St Germain, A., Paterson, C. T., Hansen, K. B., Stewert, J. W., & Toda, T. (2000). Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *American Journal of Clinical Nutrition*, *72*, 844-852.

Anderson, J. W., Johnstone, B. M., & Cook-Newell, M. E. (1995). Meta-analysis of the Effects of Soy Protein Intake on Serum Lipids. *The New England Journal of Medicine*, *333*(5), 276-282.

Anderson, N. M., Keller, S. E., Mishra, N., Pickens, S., Gradl, D., Hartter, T., Rokey, G., Dohl, C., Plattner, B., Chritel, S., & Grasso-Kelley, E. M. (2017). Salmonella Inactivation During Extrusion of an Oat Flour Model Food. *Journal of Food Science*, *82*(3), 738743. https://doi.org/10.1111/1750-3841.13629

Andino, A., & Hanning, I. (2015). Salmonella Enterica: Survival, Colonization, and Virulence Differences Among Serovars. *TheScientificWorldJournal*, *2015*. https://doi.org/10.1155/2015/520179

Balakireva, A., & Zamyatnin, A. (2016). Properties of Gluten Intolerance: Gluten Structure, Evolution, Pathogenicity and Detoxification Capabilities. *Nutrients*, *8*(10). https://doi.org/10.3390/nu8100644

Barnes, S., Boersma, B., Patel, R., Kirk, M., Darley-Usmar, V., Kim, H., & Xu, J. (2000). Isoflavonoids and chronic disease: mechanisms of action. *BioFactors (Oxford, England)*, *12*(1-4). https://doi.org/10.1002/biof.5520120133

Baylis, C. L., MacPhee, S., & Betts, R. P. (2001). Comparison of two commercial preparations of buffered peptone water for the recovery and growth of Salmonella bacteria from foods. *Journal of Applied microbiology*. https://doi.org/10.1046/j.1365-2672.2000.01145.x

BeyondMeat. (2020). Beyond Burger® - Beyond Meat - Go Beyond®.

Bianchini, A., Stratton, J., Weier, S., Hartter, T., Plattner, B., Rokey, G., Hertzel, G., Gompa, L., Martinez, B., & Eskridge, K. M. (2012). Validation of Extrusion as a Killing Step for. *Journal of Food Protection*, *75*(9), 1646-1653. https://doi.org/10.4315/0362-028X.JFP-12-085

Bianchlni, A., Stratton, J., Weier, S., Hartter, T., Plattner, B., Rokey, G., Hertzel, G., Gompa, L., Martinez, B., & Eskridge, K. M. (2012). Validation of Extrusion as a Killing Step for Enterococcus faecium in a Balanced Carbohydrate-Protein Meal by Using a Response Surface Design [Article]. *Journal of food protection*, *75*(9), 1646-1653.

BOCA. (2019). *Products*. https://www.bocaburger.com/products

Brackenridge, A. W., Katen, T. R., Klemaszewski, J. L., & Moss, D. A. (2013). *Meat substitute product*. E. P. Office. https://patents.google.com/patent/EP2645884A1/en

Brishti, F. H., Zarei, M., Muhammad, S. K. S., Ismail-Fitry, M. R., Shukri, R., & Saari, N. (2017). Evaluation of the functional properties of mung bean protein isolate for development of textured vegetable prot. *International Food Research Journal*, *24*(4), 1595-1605.

Budu-Amoako, E., Toora, S., Ablett, R. F., & Smith, J. (1992). Evaluation of the ability of primary selective enrichment to resuscitate heat-injured and freeze-injured Listeria monocytogenes cells. [Text]. *Applied and Environmental Microbiology*, *58*(9), 3177-3179.

Bullerman, L. B., & Bianchini, A. (2009). Food Safety Issues and the Microbiology of Cereals and Cereal Products. In N. L. Herefia, I. V. Wesley, & J. Santos Garcia (Eds.), *Microbiologically Safe Foods*. John Wiley & Sons Inc.

Bárta, J., Hermanová, V., & Daivis, J. (2008). Effect of Low-Molecular Additives on Precipitation of Potato Fruit Juice Protein Under Different Temperture Regimes. *Journal of Food Process Engineering*, *31*(4). https://doi.org/10.1111/j.1745-4530.2007.00167.x

Bártová, V., & Bárta, J. (2009). Chemical Composition and Nutritional Value of Protein Concentrates Isolated from Potato (Solanum tubersum L.) F*ruit Juice by Pre*cipitation with Ethanol or Ferric Chloride. *Journal of Agricultural and Food Chemistry*, *57*, 9028-9034. https://doi.org/10.1021/jf900897b

Bártová, V., Bárta, J., Brabcová, A., Zdrahal, Z., & Horackova, V. (2015). Amino acid composition and nutritional value of four cultivated South American potato species. *Journal of Food Composition and Analysis*, *20*, 78-85. https://doi.org/10.1016/j.jfca.2016.12.006

Bártová, V., Bárta, J., & Jarošova, M. (2019). Antifungal and antimicrobial proteins and peptides of potato (*Solanum tuberosum* L.) tubers and their applications. *Journal of Agricultural and Food Chemistry*, *103*, 5533-5547. https://doi.org/10.1007/s00253-019-09887-9

Capritto, A. (2020). *You can now buy the Impossible Burger at Safeway, Wegmans and more*. CNET. https://www.cnet.com/health/impossible-burger-finally-rolls-out-to-grocery-stores/

Cavallini, V., Hargarten, P. G., & Joehnke, J. (2006). *Vegetable Protein Meat Analog*.

CDC. (2008, 2019-01-31T03:14:50Z/). *Multistate Outbreak of Salmonella Agona Infections Linked to Rice and Wheat Puff Cereal (FINAL UPDATE)*. Center for Disease Control. https://www.cdc.gov/salmonella/2008/rice-wheat-puff-cereal-5-13-2008.html

CDC. (2018). National Outbreak Reporting System (NORS) Dashboard.

CDC. (2019, 2019-10-11T06:00:19Z). *Foods Most Likely to Cause Food Poisoning*. Centers for Disease Control. https://www.cdc.gov/foodsafety/foods-linked-illness.html#fruits

CDC. (2020, 2020-06-24T06:29:39Z). *Salmonella Homepage*. https://www.cdc.gov/salmonella/

Chen, F. L., Wei, Y. M., & Zhang, B. (2011). Chemical cross-linking and molecular aggregation of soybean protein duringextrusion cooking at low and high moisture content. *LWT-Food Science and Technology*, *44*, 957-962. https://doi.org/10.1016/jwt.2010.12.008

Chen, H., Zhang, Y., & Zhong, Q. (2019). Potential of acidified sodium benzoate as an alternative wash solution of cherry tomatoes: Changes of quality, background microbes, and inoculated pathogens during storage at 4 and 21°C post-washing. *Food microbiology*, *82*, 111-118. https://doi.org/10.1016/j.fm.2019.01.013

Chen, J., Zhang, L., Paoli, G. C., Shi, C., Tu, S.-I., & Shi, X. (2010). A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. *International Journal of Food Microbiology*, *137*, 168-174. https://doi.org/10.1016/j.ijfoodmicro.2009.12.004

Cheng, A. (2020). *Beyond Meat Is The Biggest Beneficiary In The Growing Plant-Based-Meat Market: Study*. Forbes. https://www.forbes.com/sites/andriacheng/2020/01/17/beyond-meat-is-the-biggest-beneficiary-in-the-growing-plant-based-meat-market-study/

D'Aoust, J. Y., Maighment, C., Stotland, P., & Boville, A. (1982). Surfactants for the Effective Recovery of Salmonella in Fatty Foods. *Journal of Food Protection*, *45*(3), 249-252.

Daquigan, N., Grim, C. J., White, J. R., Hanes, D. E., & Jarvis, K. G. (2016). Early Recovery of Salmonella from Food Using a 6-Hour Non-selective Pre-enrichment and Reformulation of Tetrathionate Broth [Text]. *frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.02103

Day, L., Augustin, M. A., Batey, I. L., & Wrigley, C. W. (2006). Wheat-gluten uses and industry needs. *Trends in Food Science & Technology*, *17*, 82-90. https://doi.org/10.1016/j.tifs.2005.10.003

Dekkers, B. L., Boom, R. M., & van der Goot, A. J. (2018). Structuring processes for meat analogues [Review Article]. *Trends in Food Science & Technology*, *81*, 25-36. https://doi.org/10.1016/j.tifs.2018.08.011

Dhayakaran, R. P. A., Neethirajan, S., Xue, J., & Shi, J. (2015). Characterization of antimicrobial efficacy of soy isoflavones against pathogenic biofilms. *Food Science and Technology*, *63*, 859-865. https://doi.org/10.1016/j.lwt/2015.04.053

Djuric, Z., Chen, G., Doerge, D. R., Heilbrun, L. K., & Kucuk, O. (2001). Effect of soy isoflavone supplementation on markers of oxidative stress in men and women. *Cancer letters*, *172*(1). https://doi.org/10.1016/s0304-3835(01)00627-9

El Youssef, C., Bonnarme, P., Fraud, S., Peron, A.-C., Helinck, S., & Landaud, S. (2020). Sensory Improvement of a Pea Protein-Based Product Using Microbial Co-Cultures of Lactic Acid Bacteria and Yeasts. *FOODS*, *9*(3). https://doi.org/10.3390/foods9030349

Fatica, M., & Schneider, K. (2011). Salmonella and produce: survival in the plant environment and implications in food safety. *Virulence*, *2*(6). https://doi.org/10.4161/viru.2.6.17880

FDA. (2002). *GRAS Notices: GRN NO.91*. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=91

FDA. (2012). *Bad Bug Book - Bad-Bug-Book-2nd-Edition-(PDF).pdf*. Food and Drug Administration. https://www.fda.gov/files/food/published/Bad-Bug-Book-2nd-Edition-%28PDF%29.pdf

FDA. (2015a). *FDA Investigated Multistate Outbreak of Salmonella Enteritidis Linked to Mung Bean Sprouts Produced by Wonton Foods Inc. of New York*. U.S. Food and Drug Administration. https://wayback.archive-it.org/7993/20170111140518/http://www.fda.gov/Food/RecallsOutbreaksEmergencies/Outbreaks/ucm424426.htm

FDA. (2015b). Rapid Method Sample Spike Production and Use. In.

FDA. (2015c). *Voluntary Recall for Navajo Pride Bleached Flour Due to Possible Health Risk*. Office of Regulatory Affairs,Office of the Commissioner. https://wayback.archive-it.org/7993/20170723005828/https://www.fda.gov/Safety/Recalls/ArchiveRecalls/2015/ucm445036.htm

FDA. (2016). *GRAS Notice 684, Mung bean protein isolate*. FDA. https://www.fda.gov/media/103224/download#:~:text=Pursuant%20to%2021%20CFR%20%C2%A7,1.3%2C%20on%20the%20basis%20of

FDA. (2018a). *Food Safety A to Z Reference Guide*. U.S. Food and Drug Administration. https://www.fda.gov/food/science-and-our-food-supply/food-safety-z-reference-guide

FDA. (2018b). *Kellogg Company Voluntarily Recalls Honey Smacks Cereal Due to Possible Health Risk | FDA*. U.S. Food and Drug Administration. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/kellogg-company-voluntarily-recalls-honey-smacks-cereal-due-possible-health-risk

FDA. (2018c). *Media Index for BAM*. U.S. Food and Drug Administration. https://www.fda.gov/food/laboratory-methods-food/media-index-bam

FDA. (2018d). Pepperidge Farm® Announces Voluntary Recall of Four Varieties of Goldfish® Crackers | FDA.

FDA. (2019a). General Mills Recalls Five Pound Bags of Gold Medal Unbleached Flour | FDA.

FDA. (2019b). Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds. In (3.0 ed.): U.S Food and Drug Administration.

FDA. (2019c). *Hometown Food Company Recalls Two Production LOT Codes of Pillsbury® Unbleached All-Purpose 5lb Flour Due to Possible Health Risk | FDA*. U.S. Food and Drug Administration. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/hometown-food-company-recalls-two-production-lot-codes-pillsburyr-unbleached-all-purpose-5lb-flour

FDA. (2020a). *BAM Chapter 5 Salmonella*. U.S. Food and Drug Administration. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-5-salmonella#Refs

FDA. (2020b). *Food Allergies*. FDA. https://www.fda.gov/food/food-labeling-nutrition/food-allergies

FDA. (2020c). *Sunshine Mills, Inc. Issues Voluntary Recall of Nature's Menu® Super Premium Dog Food with a Blend of Real Chicken & Quail Because of Possible Salmonella Health Risk | FDA*. U.S. Food and Drug Administration. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/sunshine-mills-inc-issues-voluntary-recall-natures-menur-super-premium-dog-food-blend-real-chicken

FDA. (2020d). *The Quaker Oats Company Issues Voluntary Recall of a Small Quantity of Cap'n Crunch's Peanut Butter Crunch Cereal Distributed to Five Target Stores Due to Possible Health Risk | FDA*. U.S. Food and Drug Administration. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/quaker-oats-

company-issues-voluntary-recall-small-quantity-capn-crunchs-peanut-butter-crunch-cereal

FDA. (2021). *Natural Balance Pet Foods, Inc. Voluntarily Recalls L.I.D. Green Pea & Chicken Dry Cat Formula for Possible Salmonella Contamination | FDA*. U.S. Food and Drug Administration. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/natural-balance-pet-foods-inc-voluntarily-recalls-lid-green-pea-chicken-dry-cat-formula-possible

Fett, W. F., & Cooke, P. H. (2003). Scanning electron microscopy of native biofilms on mung bean sprouts. *Journal of Microbiology*, *49*, 45-50. https://doi.org/10.1139/W03-002

Filho, P., F. Souza, Ramkumar, B. N., Andersson, Patrik, R. L., & Mohammad, J. T. (2018). Vegan-mycoprotein concentrate from pea-processing industry byproduct using edible filamentous fungi [article]. *Fungal Biology and Biotechnology*, *5*(1), 1-10. https://doi.org/10.1186/s40694-018-0050-9

Finnigan, T. J. A., Wall, B. T., Wilde, P. J., Stephens, F. B., Taylor, S. L., & Freedman, M. R. (2019). Mycoprotein: The Future of Nutritious Nonmeat Protein, a Symposium Review. *Curr Dev Nutr*, *3*(6). https://doi.org/10.1093/cdn/nzz021

Foster, J. W., & Hall, H. K. (1991). Inducible pH Homesostasis and the Acid Tolerance Response of *Salmonella typhimurium*. *Journal of Bacteriology*, *173*(16), 5129-5135.

Fu, T.-J., Reineke, K. F., Chirtel, S., & VanPelt, O. (2008). Factors Influencing the Growth of Salmonella during Sprouting of Naturally Contaminated Alfalfa Seeds. *Journal of Food protection*, *71*(5), 888-896.

Gaan, K. (2021). *2020 State of the Industry Report: Plant-based Meat, Eggs, and Dairy*. T. G. F. Institute.

Galié, S., García-Gutiérrez, C., Miguélez, E. M., Villar, C. J., & Lombó, F. (2018). Biofilms in the Food Industry: Health Aspects and Control Methods. *Front Microbiol*, *9*. https://doi.org/10.3389/fmicb.2018.00898

Gardein. (2020). *Gardein*. https://www.gardein.com/

Geistlinger, T. (2015). *Plant based meat structured protein products*.

Gorissen, S. H. M., Crombag, J. J. R., Senden, J. M. G., Waterval, W. A. H., Bierau, J., Verdijk, L. B., & van Loon, L. J. C. (2018). Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino Acids*, *50*.

Grzebinski, T. (2020). *Beyond Milk and Meat: Plant-Based Foods Continue to Sprout New Growth Across Categories.* IRI. https://www.iriworldwide.com/en-US/Insights/Blog/Beyond-Milk-and-Meat-Plant-Based-Foods-Continue-to

Gupta, R. S., Warren, C. M., Smith, B. M., Jiang, J., Blumenstock, J. A., Davis, M. M., Schleimer, R. P., & Nadeau, K. C. (2019). Prevalence and Severity of Food Allergies Among US Adults. *JAMA Network Open*, *2*(1).

GVR. (2020). *Plant-based Meat Market Growth & Trends Report, 2020-2027*. https://www.grandviewresearch.com/industry-analysis/plant-based-meat-market

Gómez, I., Ibañez, F. C., & Beriain, M. J. (2019). Physicochemical and sensory properties of sous vide meat and meat analog products marinated and cooked at different temperature-time combinations [article]. *International Journal of Food Properties*, *22*(1), 1693-1708. https://doi.org/10.1080/10942912.2019.1666869

Hammack, T. S., Amaguaña, M. R., & Andrews, W. H. (2001). An Improved Method for the Recovery of *Salmonella* Serovars from Orange Juice using Universal Preenrichment Broth. *Journal of Food Protection*, *64*(5), 659-663.

Hammack, T. S., Jacobson, A. P., & Andrews, W. H. (2008). The Effect of Preenrichment and Selective Enrichment Meida on Recovery of *Salmonella* Typhi from the Tropical Fruit Mamey. *Journal of AOAC International, 91*.

Hashempour-Baltork, F., Hosseini, S. M., Assarehzadegan, M.-A., Khosravi-Darani, K., & Hosseini, H. (2020). Safety assays and nutritional values of mycoprotein produced by Fusarium venenatum IR372C from date waste as substrate. *Journal of the Science of Food and Agricultire*. https://doi.org/10.1002/jsfa.10483

Hashempour-Baltork, F., Khosravi-Darani, K., Hosseini, H., Farshi, P., & Reihani, S. F. S. (2020). Mycoproteins as safe meat substitutes. *Journal of Cleaner Production*, *253*. https://doi.org/10.1016/j.clepro.2020.119958

Hintz, T., Matthews, K. K., & Di, R. (2015). The Use of Plant Antimicrobial Compounds for Food Preservation [Text]. https://doi.org/10.1155/2015/246264

Hwang, K.-A., & Choi, K.-C. (2015). Anticarcinogenic Effects of Dietary Phytoestrogens and Their Chemopreventive Mechanisms [Article]. *Nutrition & Cancer*, *67*(5), 796-803. https://doi.org/10.1080/01635581.2015.1040516

Ismail, I., Hwang, Y.-H., & Joo, S.-T. (2020). Meat analog as future food: a review. *Journal of Animal Science and Technology*, *62*(2), 111-120.

Jacobson, A. P., Wang, H., Gill, V. S., Duvall, R., Arce, G., Chirtel, S., & Hammack, T. S. (2017). Relative effectiveness of selected preencirhment media for the detection of *Salmonella* from leafy green produce and herbs. *Food Microbiology*, *63*, 123-128. https://doi.org/https://doi.org/10.1016/j.fm.2016.11.006

Jiang, I. (2019). Plant-based 'meat' is conquering fast food. Here's where you can get meat substitutes like the Beyond Burger and the Impossible Taco.

https://www.businessinsider.com/burger-king-tgi-fridays-chains-sell-plant-based-meat-2019-5

Jin, Z., Yang, Y. X., Choi, J. Y., Shinde, P. L., Yoon, S. Y., Hahn, T. W., Lim, H. T., Park, Y. K., Hahm, K. S., Joo, J. W., & Chae, B. J. (2008). Effect of potato (Solanum tubersum L. cv. Golden valley) protein having antimicrobial activity on the growth performance and intestinal microflora and morphology in weanling pigs. *Animal Feed Science and Technology*, *140*, 139-154. https://doi.org/10.1016/j.anifeedsci.2007.12.006

Keerthirathne, T. P., Ross, K., Fallowfield, H., & Whiley, H. (2016). A Review of Temperature, pH, and Other Factors that Influence the Survival of Salmonella in Mayonnaise and Other Raw Egg Products. *Pathogens*, *5*(4). https://doi.org/10.3390/pathogens5040063

Keeton, J. T., & Melton, C. C. (1978). Factors associated with microbial growth in ground beef extended wtih varying levels of texture soy protein. *Journal of Food Science*, *43*(4). https://doi.org/10.1111/j.11365-2621.1978.tb15250.x

Kelley, T. R., & Walker, P. M. (1999). Bacterial concentration reduction of food waste amended animal feed using a single-screw dry-extrusion process. *Bioresource Technology*, *67*, 247-253. https://doi.org/10.1016/S0960-8524(98)00118-7

Kim, K., Choi, B., Lee, I., Lee, H., Kwon, S., Oh, K., & Kim, A. Y. (2011). Bioproduction of mushroom mycelium of Agaricus bisporus by commercial submerged fermentation for the production of meat analogue. *Journal of the Science of Food and Agriculture*, *91*(9). https://doi.org/10.1002/jsfa.4348

Knicky, M., & Spörndly, R. (2015). Short communication: Use of a mixture of sodium nitrite, sodium benzoate, and potassium sorbate in aerobically challenged silages. *Journal of dairy science*, *98*(8), 5729-5734. https://doi.org/10.3168/jds.2015-9332

Knolder, L. A., & Elfencein, J. R. (2019). *Salmonella enterica*. *Trends in Microbiology*, *27*(11). https://doi.org/10.1016/j.tim.2019.05.002

Koyuncu, S., & Häggblom , P. (2009). A comparative study of cultural methods for the detection of Salmonella in feed and feed ingredients [Text]. *BMC Veterinary Research*, *5*. https://doi.org/10.1186/1746-6148-5-6

Krintiras, G. A., Göbel, J., van der Goot, A. J., & D., S. G. (2015). Production of structured soy-based meat analogues using simple shear and heat in a Couette Cell. In (Vol. 160, pp. 34-41).

Kumar, P., Chatli, M. K., Mehta, N., Parminder, S., Malav, O. P., & Verma, A. K. (2015). Meat analogues: Health promising sustainable meat subsitutes. *Critical Reviews in Food Science and Nutrition*, *57*(5), 923-932. https://doi.org/10.1080/10408398.2014.939739

Kumar, P., Kumar, R. R., & Sharma, B. D. (2012). Optimization of the Level of Wheat Gluten in Analogue Meat Nuggets. *Indian Journal of Veterinary Research*, *21*(1), 54-59.

Kumar, S., & Pandey, G. (2020). Biofortification of pulses and legumes to enhance nutrition [Review Article]. *Heliyon*, *6*(3). https://doi.org/10.1016/j.heliyon.2020.e03682

Lachman, J., Hamouz, K., Dvorak, P., & Orsak, M. (2005). The effect of selected factors on the content of protein and nitrates in potato tubers. *Plant, Soil and Environment*, *51*(10), 431-438.

Lamartiniere, C. A., Cotroneo, M. S., Fritz, W. A., Wang, J., Mentor-Marcel, R., & Elgavish, A. (2002). Genistein Chemoprevention: Timing and Mechanisms of Action in Murine Mammary and Prostate. *The Journal of Nutrition*, *132*(3), 552S-558S. https://doi.org/10.1093/jn/132.3.552S

Landry, K. S., Chang, Y., McClements, D. J., & McLandsborough, L. (2014). Effectiveness of a novel spontaneous carvacrol nanoemulsion against *Salmonella enterica* Enteritidis and *Escherichia coli O157:H7* on contaminated mung bena and alfalfa seeds. *International Journal of Food Microbiology*, *187*, 15-21. https://doi.org/10.1016/j.ijfoodmicro.2014.06.030

Li, T., Huo, X.-N., Zhu, K.-X., & Zhou, H.-M. (2018). Effects of alkali on protein polymerization and textural characteristics of textured wheat protein. *Food Chemistry*, 579-587. https://doi.org/10.1016/j.foodchem.2017.06.155

Liao, C.-H., & Fett, W. F. (2003). Isolation of *Salmonella* from alfalfa seed and demonstration of imparied growth of heat-injured cells in seed homogenates. *International Journal of Food Microbiology*, *82*(3), 245-253. https://doi.org/https://doi.org/10.1016/S0168-1605(02)00309-4

Liao, C. H., & Fett, W. F. (2005). Resuscitation of acid-injured Salmonella in enrichment broth, in apple juice and on the surfaces of fresh-cut cucumber and apple. *Letters in Applied Microbiology*, *41*, 487-492. https://doi.org/doi:10.1111/j.1472-765X.2005.01794.x

Lin, S., Huff, H. E., & Hsieh, F. (2000). Texture and Chemical Characteristics of Soy Protein Meat Analog Extruded at High Moisture. *Journal of Food Science*, *65*(2). https://doi.org/10.1111/j.1365-2621.2000.tb15991.x

Lu, Z. X., He, J. F., Zhang, Y. C., & Bing, D. J. (2019). Composition, physicochemical properties of pea protein and its application in functional foods. *Critical reviews in food science and nutrition*. https://doi.org/10.1080/10408398.2019.1651248

Lupo, L. (2019). The Impossible Burger. *Quality Assurance and Food Safety Magazine*.

Margot, H., Zwietering, M. H., Joosten, H., O'Mahony, E., & Stephan, R. (2015). Evaluation of different buffered peptone water (BPW) based enrichment broths for dection of Gram-negative foodborne pathogens from various food matrices. *International Journal of Food Microbiology*, *215*, 109-115. https://doi.org/10.1016/j.ijfoodmicro.2015.07.033

Mathai, J. K., Liu, Y., & Stein, H. H. (2017). Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS) | British Journal of Nutrition | Cambridge Core. *British Journal of Nutrition*, *117*(4). https://doi.org/doi:10.1017/S0007114517000125

McCallum, L., Paine, S., Sexton, K., Dufour, M., Dyet, K., Wilson, M., Campbell, D., Bandaranayake, D., & Hope, V. (2013). An Outbreak of Salmonella Typhimurium Phage Type 42 Associated With the Consumption of Raw Flour. *Foodborne pathogens and disease*, *10*(2). https://doi.org/10.1089/fpd.2012.1282

MeticulousMarketResearch. (2020, 12/2/2020). *Meat Substitute Market to Reach $17.5 Billion by 2027,*. globenewswire. https://www.globenewswire.com/news-release/2020/12/02/2138084/0/en/Meat-Substitute-Market-to-Reach-17-5-Billion-by-2027-Growing-at-a-CAGR-of-15-1-from-2020-With-COVID-19-Impact-Meticulous-Research-Analysis.html

Millar, K. A., Gallagher, E., Burke, R., McCarthy, S., & Barry-Ryan, C. (2019). Proximate composition and anti-nutritional factors of fava-bean (Vicia faba), green-pea and yellow-pea (Pisum sativum) flour [Article]. *Journal of Food Composition and Analysis*, *82*. https://doi.org/10.1016/j.jfca.2019.103233

Moghadam, M., Salami, M., Mohammadian, M., Khodadadi, M., & Emam-Djomeh, Z. (2020). Development of antioxidant edible films based on mung bean protein enriched with pomegranate peel. *Food Hydrocolloids*, *104*. https://doi.org/10.1015/j.foodhyd.2020.105735

Mohle-Boetani, J., Farrar, J., Bradley, P., Barak, J., Miller, M., Mandrell, R., Mead, P., Keene, W., Cummings, K., Abbott, S., & Werner, S. (2009). Salmonella infections associated with mung bean sprouts: epidemiological and environmental investigations. *Epidemiology and infection*, *137*(3). https://doi.org/10.1017/S0950268808000411

MorningStarFarms. (2020). *MorningStar Farms® Grillers® Original Veggie Burgers*. https://www.morningstarfarms.com/en_US/products/burgers.html

Murata, M., Midorikawa, K., Koh, M., Umezawa, K., & Kawanishi, S. (2004). Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs. *Biochemistry*, *43*(9). https://doi.org/10.1021/bi035613d

Murphy, P. A., Barua, K., & Hauck, C. C. (2002). Solvent extraction selection in the determination of isoflavones in soy foods. *Journal of Chromatography*, *777*, 129-138.

Myoda, S. P., Gilbreth, S., Akins-Lewenthal, D., Davidson, S. K., & Samadpour, M. (2019). Occurrence and Levels of Salmonella, Enterohemorrhagic Escherichia coli, and Listeria in Raw Wheat. *Journal of Food Protection*, *82*(6), 1022-1027. https://doi.org/10.4315/0362-028X.JFP-18-345

Nagarajan, S. (2020). *Starbucks ties up with Impossible Foods to sell a new plant-based breakfast sandwich on revamped summer menu*. Business Insider.

https://www.businessinsider.com/starbucks-partners-with-impossible-new-plant-based-sandwich-2020-6

NBCF. (2021). *Risk Factors*. National Breast Cancer Foundation. https://www.nationalbreastcancer.org/breast-cancer-risk-factors/

NCI. (2017, 01/17/2014 - 07:00). *Antioxidants and Cancer Prevention - National Cancer Institute*. National Cancer Institute. https://www.cancer.gov/about-cancer/causes-prevention/risk/diet/antioxidants-fact-sheet

Neogen. (2021). *Solutions for Food and Animal Safety*. https://www.neogen.com/

Nestle. (2019). *Nestlé launches new plant-based burgers and grounds in US, Switzerland*. @nestle. https://www.nestle.com/media/news/nestle-launch-plant-based-burgers-grounds-us-switzerland

Nsoesie, E., Kluberg, S. A., & Brownstein, J. S. (2014). Online reports of foodborne illness capture foods implicated in official foodborne outbreak reports. *Preventative Medicine*, *67*, 264-269. https://doi.org/10.1016/j.ypmed.2014.08.003

O'Donnell, K., Cigelnik, E., & Casper, H. (1998). Molecular phylogenetic, morphological, and mycotoxin data support reidentification of the Quorn mycoprotein fungus as Fusarium venenatum. *Fungal genetics and biology : FG & B*, *23*(1). https://doi.org/10.1006/fgbi.1997.1018

Oikawa, S. Y., Bahniwal, R., Holloway, T. M., Lim, C., McLeod, J. C., McGlory, C., Baker, S. K., & Phillips, S. M. (2020). Potato Protein Isolate Stimulates Muscle Protein Synthesis at Rest and with Resistance Exercise in Young Women. *Nutrients*, *12*(5). https://doi.org/10.3390/nu12051235

Okekunle, A. P., Gao, J., Wu, X., Feng, R., & Sun, C. (2020). Higher dietary soy intake appears inversely related to breast cancer risk independent of estrogen receptor breast cancer phenotypes [Article]. *Heliyon*, *6*(7). https://doi.org/10.1016/j.heliyon.2020.e04228

Oliveira, M. G. d. C., Bassinello, P. Z., Lobo, V. L. d. S., & Rinaldi, M. M. (2012). Stability and microbiological quality of rice bran subjected to different heat treatments / Estabilidade e qualidade microbiológica de farelo de arroz submetido a diferentes tratamentos térmicos [research article]. *Food Science and Technology*, *32*(4), 725-733. https://doi.org/10.1590/S0101-20612012005000095

Patel, R., Boersma, B., Crawford, J., Hogg, N., Kirk, M., Kalyanaraman, B., Parks, D., Barnes, S., & Darley-Usmar, V. (2001). Antioxidant mechanisms of isoflavones in lipid systems: paradoxical effects of peroxyl radical scavenging. *Free radical biology & medicine*, *31*(12). https://doi.org/10.1016/s0891-5849(01)00737-7

PHAC. (2017, 2017-01-13). *Public Health Notice - Outbreak of E. coli infections linked to various flours and flour products - Canada.ca*. Public Health Agency of Canada. https://www.canada.ca/en/public-health/services/public-health-notices/2017/public-health-notice-outbreak-e-coli-infections-linked-various-flours-flour-products.html

Pietsch, V. L., Emin, A., & Schuchmann, H. P. (2017). Process conditions influencing wheat gluten polymerization during high moisture extrusion of meat analog products. *Journal of Food Engineering*, *198*, 28-25. https://doi.org/10.1016/j.jfoodeng.2016.10.027

Pietsch, V. L., Karbstein, H. P., & Emin, M. A. (2018). Kinetics of wheat gluten polymerization at extrusion-like conditions relevant for the production of meat analog products [Article]. *Food Hydrocolloids*, *85*, 102-109. https://doi.org/10.1016/j.foodhyd.2018.07.008

Pietsch, V. L., Werner, R., Karbstein, H. P., & Emin , M. A. (2019). High moisture extrusion of wheat gluten: Relationship between process parameters, protein polymerization, and final product characteristics. *Journal of Food Engineering*, *259*, 3-11. https://doi.org/10.1016/j.jfoodeng.2019.04.006

Pina-Perez, M. C., & Perez, M. A. F. (2018). Antimicrobial potential of legume extracts against foodborne pathogens: A review. *Trends in Food Science and Technology*, *72*, 114-124. https://doi.org/10.1016/j.tifs.2017.12.007

Polak, R., M., P. E., & Campbell, A. (2015). Legumes: Health Benefits and Culinary Approaches to Increase Intake [Text]. https://doi.org/10.2337/diaclin.33.4.198

Quorn. (2018). *Quron Footprint Comparison Report*. https://www.quorn.us/files/content/Carbon-Trust-Comparison%20Report-2018.pdf

Quorn. (2020a). *Mycoprotein*. QuornFoods. https://www.quorn.us/mycoprotein

Quorn. (2020b). *The Story of Quorn Foods*. @QuornFoods. https://www.quorn.us/company

Randhir, R., Lin, Y.-T., & Shetty, K. (2004). Stimulation of phenolics, antioxidant, and antimicrobial activities in dark germinated mung bean sprouts in response to peptide and phytochemical elicitors. *Process Biochemistry*, *39*, 637-646.

RCoreTeam. (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing. In. Vienna, Australia.

Reed, E., Ferreira, C. M., Bell, R., Brown, W., E., & Zheng, J. (2018). Plant-Microbe and Abiotic Factors Influencing Salmonella Survival and Growth on Alfalfa Sprouts and Swiss Chard Microgreens [Text]. https://doi.org/10.1128/AEM.02814-17

Rehman, S., & Khanum, A. (2011). Isolation and characterization of peptide(s) from Pisum sativum having antimicrobial activity against various bacteria. *Pakistan Journal of Botany*, *43*(6), 2971-2978.

Reinicke, C. (2019). *Beyond Meat's blockbuster year has ignited the plant-based food industry. Here are 6 companies that have launched fake 'meat' products to compete*. https://markets.businessinsider.com/news/stocks/top-6-beyond-meat-competitors-made-by-traditional-food-companies-2019-9-1028506115#5-conagra-brands5

Rizzo, G., & Baroni, L. (2018). Soy, Soy Foods and Their Role in Vegetarian Diets [Text]. https://doi.org/10.3390/nu10010043

Rosen, D. K., Gallardo, M., Vail, M., & Hellberg, R. S. (2020). Microplate immunocapture coupled with the 3M molecular detection system and selective plating for rapid detection of *Salmoenlla* infantis in dry dog food treats. *Journal of Microbiological Methods*, *172*. https://doi.org/10.1016/j.mimet.2020.105881

Samard, S., & Ryu, G. H. (2019). A comparison of physicochemical characteristics, texture, and structure of meat analogue and meats. *J Sci Food Agric*, *99*(6), 2708-2715. https://doi.org/10.1002/jsfa.9438

Settembre, J. (2019). *Good news for Beyond Meat? 95% of people who order vegan burgers when dining out aren't even vegetarians*. Marketwatch. https://www.marketwatch.com/story/more-meat-eaters-are-ordering-plant-based-burgers-when-dining-out-2019-07-17

Sha, L., & Xiong, Y. L. (2020). Plant protein-based alternatives of reconstructed meat: Science, technology, and challenges. *Trends in Food Science and Technology*, *102*, 51-61. https://doi.org/10.1016/j.tifs.2020.05.022

Shanker, D. (2021, 2021-04-16T10:00:39.969Z). *Impossible and Beyond Slash Prices as Fake-Meat Market Heats Up*. @BW. https://www.bloomberg.com/news/articles/2021-04-16/beyond-meat-bynd-impossible-foods-battle-over-future-of-fake-meat-industry

Shewry, P. R. (2009). Wheat. *Journal of Experimental Botany*, *60*(6), 1527-1553. https://doi.org/10.1093/jxb/erp058

Singh, S., Gamlath, S., & Wakeling, L. (2007). Nutritional aspects of food extrusion: a review. *International Journal of Food Science and Technology*, *42*(8). https://doi.org/10.1111/j.1365-2621.2006.01309.x

Sitohy, M., Mahgoub, S., Osman, A., El-Masry, R., & Al-Gaby, A. (2013). Extent and mode of action of cationic legume proteins against *Listeria monocytogenes* and *Salmonella* enteritidis. *Probiotics and Antimicrobial Proteins*, *5*, 195-205.

Smadi, H., Sargeant, J. M., Shannon, H. S., & Raina, P. (2012). Growth and inactivation of *Salmonella* at low refrigerated storage temperatures and thermal inactivation on raw chicken meat and laboratory media: mixed effect meta-analysis. *Journal of Epidemiology and Global Health*, *2*, 165-179. https://doi.org/10.1016/j.jegh.2012.12.001

Suman, G., Nupur, M., Anuradha, S., & Pradeep, B. (2015). Single Cell Protein Production: A Review. *International Journal of Current Microbiology and Applied Sciences*, *4*(9), 251-261.

Tang, D., Dong, Y., Ren, H., Li, L., & He, C. (2014). A review of phytochemistry, metabolite changes, and medicinal uses of the common food mung bean and its sprouts (Vigna radiata). In *Chem Cent J* (Vol. 8, pp. 4). https://doi.org/10.1186/1752-153x-8-4

Taskila, S., Tuomola, M., & Ojamo, H. (2012). Enrichment cultivation in detection of food-borne *Salmonella*. *Food control*, *26*(2), 369-377. https://doi.org/10.1016/j.food.cont.202.01.043

Trinci, A. P. (1994). Evolution of the QuornB myco-protein fungus, Fusarium graminearum A315. *Microbiology (Reading, England)*, *140*, 2181-2188. https://doi.org/10.1099/13500872-140-9-2181

Trottet, G., Fenandes, S., Grünz, G., Thoma, J. P., & Brunner-Komorek, K. (2016). *A process for preparing meat-analogue foor product*.

Upadhyaya, S., Tiwari, S., Arora, N. k., & Singh, D. P. (2016). Microbial Protein: A Valuable Component for Food Security. In *Microbes and Environmental Management* (pp. 259-279).

Verma, T., & Subbiah, H. (2019). Conical twin-screw extrusion is an effective inactivation process for Salmonella in low-moisture foods at temperatures above 65 °C. *LWT- Food Science and Technology*, *114*. https://doi.org/doi.org/10.1016/j.lwt.2019.108369

Vogel, K. P., Johnson, V. A., & Mattern, P. J. (1978). Protein and Lysine Contents of Endosperm and Bran of the Parents and Progenies of Crosses of Common Wheat - 189480033.pdf. *Publications from USDA-ARS / UNL Faculty*.

Waglay, A., & Karboune, S. (2020). Predictive Consumer Acceptance Models and Quality Attributes for Cookies Enriched with Potato Protein Isolate and Concentrate [OriginalPaper]. *Food and Bioprocess Technology*, 1-16. https://doi.org/doi:10.1007/s11947-020-02508-8

Wang, H., Gill, V. S., Cheng, C.-M., Gonzalez-Escalona, N., Irvin, K. A., Zheng, J., Bell, R. L., Jacobson, A. P., & Hammack, T. S. (2015). Evaluation and comparison of rapid methods

for the detection of *Salmonella* in naturally contaminated pine nuts using different preenrichment media. *Food Microbiology*, *46*, 58-65. https://doi.org/https://doi.org/10.1016/j.fm.2014.06.028

Wiebe, M. (2002). Myco-protein from Fusarium venenatum : a well-established product for human consumption [OriginalPaper]. *Applied Microbiology and Biotechnology*, *58*(4), 421-427. https://doi.org/doi:10.1007/s00253-002-0931-x

Wiebe, M. (2004). Quorn™ Myco-protein - Overview of a successful fungal product. (1), 17.

Wierup, M., & Häggblom, P. (2010). An Assessment of Soybeans and Other Vegetable Proteins as Source of Salmonella Contamination in Pig Production. *Acta veterinaria Scandinavica*, *52*(1). https://doi.org/10.1186/1751-0147-52-15

Wilder, A. (2021, 2021-02-15). *Gates-Backed Nature's Fynd Unveils Initial Products Made From Fermented Fungi*. TheSpoonTech. https://thespoon.tech/gates-backed-natures-fynd-unveils-initial-products-made-from-fermented-fungi/

Wong, K. M., Corradini, M. G., Autio, W., & Kinchla, A. J. (2019). Sodium reduction strategies through use of meat extenders (white button mushrooms vs. textured soy) in beef patties. In *Food Sci Nutr* (Vol. 7, pp. 506-518). https://doi.org/10.1002/fsn3.824

Xia, J. a., Song, H., Huang, K., Li , S., & Guan, X. (2020). Purification and characterization of antioxidant peptides from enzymatic hydrolysate of mungbean protein - Xia - 2020 - Journal of Food Science - Wiley Online Library. *Journal of Food Science*. https://doi.org/10.1111/1750-3841.15139

Yi-Shen, Z., Shuai, S., & FitzGerald, R. (2018). Mung bean proteins and peptides: nutritional, functional and bioactive properties. In *Food and Nutrition Research* (Vol. 62). https://doi.org/10.29219/fnr.v62.1290

Young-deok, K. (2007). *A method of producing a mycoprotein, a mycoprotein produced thereby, a low-calorie meat substitute, a meat flavor and a meat flavor enhancer made of the same mycoprotein*. https://patents.google.com/patent/KR100762848B1/en

Yuliarti, O., Kovis, T. J. K., & Yi, N. J. (2021). Structuring the meat analogue by using plant-based derived composites. *Journal of Food Engineering*, *288*.

Zaheer, K., & Akhtar, M. H. (2017). An updated review of dietary isoflavones: Nutrition, processing, bioavailability and impacts on human health [research-article]. *Critical Reviews in Food Science and Nutrition*, *57*(6). https://doi.org/10.1080/10408398.2014.989958

Zhang, G., Brown, E. W., & Hammack, T. S. (2013). Comparison of different preenrichment broths, egg:preenrichment broth ratios, and surface disinfection for the detection of *Salmonella enterica* ssp. *enterica* serovar Enteritidis in shell eggs. *Poultry Science*, *92*(11), 3010-3016. https://doi.org/https://doi.org/10.3382/ps.2013-03022

Zhang, G., L, M., Patel, N., Swaminathan, B., Wedel, S., & Doyle, M. (2007). Isolation of Salmonella typhimurium from outbreak-associated cake mix. *Journal of food protection*, *70*(4). https://doi.org/10.4315/0362-028x-70.4.997

Zheng, Q., Mikš-Krajnik, M., D'Souza, C., Yang, Y., Heo, D., Kim, S., Lee, S., & Yuk, H. (2015). Growth of Healthy and Sanitizer-Injured Salmonella Cells on Mung Bean Sprouts in Different Commercial Enrichment Broths. *Food microbiology*, *52*. https://doi.org/10.1016/j.fm.2015.07.013

Zokaityte, E., Lele, V., Starkute, V., Zavistanaviciute, P., Klupsaite, D., Bartkevics, V., Pugajeva, I., Bērziņa, Z., Gruzauskas, R., Sidlauskiene, S., Juodeikiene, G., Santini, A., & Bartkiene, E. (2021). The influence of combined extrusion and fermentation processes

on the chemical and biosafety parameters of wheat bran [Article]. *LWT*, *146*. https://doi.org/10.1016/j.lwt.2021.111498