PRESENCE OF *ESCHERICHIA COLI* AND *SALMONELLA ENTERICA* ON WHEAT AND

POSSIBLE CONTROL MEASURES

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North Dakota State University's regulations and meets the accepted

standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Wheat (*Triticum* spp.) is one of the most important cereal crops grown in the U.S., with an average of 50 million acres planted on an annual basis. Wheat is milled into flour or semolina, which is used to make bread, cookies, noodles, and pasta. Because some consumers eat raw flour, it is necessary for it to be free of pathogens including *Escherichia coli* and *Salmonella enterica*. The fecal matter of cattle and poultry often contains these bacteria and can contaminate wheat. Currently, there are no requirements for controlling *E. coli* and *S. enterica* in wheat, which has resulted in outbreaks of both pathogens. Thus, future research must focus on the development of processes that control these pathogens in wheat. Possibilities include feeding livestock probiotics, pasteurization, irradiation, and non-thermal plasma processing. The further development and implementation of these processes would decrease the safety risks associated with consuming raw wheat.

ACKNOWLEDGEMENTS

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Lastly, I would like to express my endless gratitude to my parents for their unfailing support and incessant encouragement throughout my academic career. All my accomplishments during this time would not have been possible without your support. Thank you for all you have done for me.

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LIST OF ABBREVIATIONS

Stx1 ....Shiga toxin type 1

Stx2 ....Shiga toxin type 2

Gb3...Globotriaosylceramide

Gb4...Globotetraosylceramide

CFU...Colony forming units

HACCP ....Hazard analysis and critical control point

GENERAL INTRODUCTION

Wheat (*Triticum* spp.) is a cereal grain planted on an average of 50 million acres of land

every year in the U.S. (United States Department of Agriculture Economic Research Service

2017b). There are six classes of wheat produced in the U.S. including hard red spring wheat

(*Triticum aestivum* L.), hard red winter wheat (*Triticum aestivum* L.), hard white wheat (*Triticum*

aestivum L.), soft red winter wheat (*Triticum aestivum* L.), soft white wheat (*Triticum aestivum*

L.), and durum wheat (*Triticum durum* Desf.). Each class of wheat has unique uses from cookies

to spaghetti, and each class is grown in different regions of the U.S. Wheat flour and semolina

(the products of milling wheat) are also used as ingredients in numerous products not commonly

associated with wheat, including candy and sauces. Despite the numerous end uses, all wheat is

currently grown outdoors and is exposed to pathogenic microorganisms including *Escherichia*

coli and *Salmonella enterica* (Berghofer et al. 2003). This exposure can result in foodborne

illness when flour or semolina is consumed without being properly cooked or baked.

E. coli and *S. enterica* are gram negative, rod-shaped, non-spore forming, facultative

anaerobic bacteria that are practically ubiquitous in the environment (Government of Canada

2014; 2011). Both of these bacteria are *Enterobacteriaceae* that cause illness in both humans and

animals (i.e. they are zoonotic). There are seven serovars of *E. coli* that are of the upmost

concern including O26, O45, O103, O111, O121, O145, and O157 because these serovars cause

the majority of foodborne illnesses (Centers for Disease Control and Prevention 2015). These

serovars of *E. coli* produce Shiga toxins when ingested by humans, and these toxins cause

illnesses of varying degrees of severity from mild diarrhea to hemolytic uremic syndrome. One

of the serovars of *S. enterica* that causes the majority of foodborne illness is Typhimurium

(Centers for Disease Control and Prevention 2016b). This serovar of *S. enterica* colonizes the intestines of humans when ingested and causes enterocolitis (Coburn et al. 2007).

Both *E. coli* and *S. enterica* colonize the digestive systems of livestock, which typically serve as the main host of these pathogenic bacteria (Winfield and Groisman 2003). These livestock animals shed these pathogens in their feces, which can contaminate nearby soil and water. Fields of wheat can be contaminated by the fecal matter in water, soil, and manure-based fertilizers. These pathogens can survive outside their hosts for prolonged amounts of time, which results in extended time-periods during which they can contaminate fields of wheat. *E. coli* can survive in sediment for 1.5 days and in soil for up to one week. *S. enterica* can survive in manure-based fertilizer for up to 21 days and in soil for up to one year. In addition, vectors including birds and flies can efficiently spread *S. enterica* over a wide geographic area within a short amount of time. Due to the numerous possible routes of *E. coli* and *S. enterica* contamination on wheat, about 1 % of wheat samples are typically contaminated by one or both of these pathogens (Berghofer et al. 2003; Richter et al. 1993).

Currently, control measures for these two pathogens are seldom utilized prior to milling wheat into flour or semolina. However, there are numerous control measures that could be utilized in the future provided more research is performed to demonstrate their efficacy. One proactive control measure that could be implemented is feeding livestock probiotics that compete with *E. coli* and *S. enterica* to decrease the levels of these pathogens shed in fecal matter. In addition, pasteurization, irradiation, and non-thermal plasma processing are reactive control measures that could be utilized to inactivate *E. coli* and *S. enterica* already on wheat prior to milling.

Regardless of the control measure implemented, the quality of the wheat must not be negatively affected by said control measure. If the control measure is detrimental to the quality of the wheat, it is unlikely that a miller would implement it. However, the lower temperature ranges of some of the control measures previously mentioned should maintain the functionality of the starch and protein in the wheat so that end use quality would not be sacrificed in exchange for an increase in safety. The main components of wheat quality that must be assessed following the application of a control measure are starch and protein (i.e. gluten) functionality. The functionality of starch in pasteurized wheat can be assessed using rapid viscosity analysis and determining the falling number of the wheat (U.S. Wheat Associates 2017). The functionality of protein in pasteurized wheat can be assessed using wet gluten analysis and by determining the mixing profile of the flour with water using a farinograph. Utilizing these quality assessments will ensure that control measures utilized maintain the functionality of the starch and protein in the wheat.

Implementation of a control measure for *E. coli* and *S. enterica* in wheat prior to milling would effectively reduce the risk of foodborne illness from consumption of completely raw or improperly processed wheat products. This reduction of risk would lower the economic burden of the associated foodborne illnesses. While the implementation costs of this control measure will exist, costs associated with recalls would be greatly reduced (if not eliminated) (Golan et al. 2000). In addition, costs to consumers would be minimized (Scharff 2015). Thus, the implementation of a control measure would lead to an overall increase in the level of safety associated with consuming wheat products and a decreased economic burden.

LITERATURE REVIEW

Wheat

Wheat (*Triticum* spp.) kernels, such as the one shown in Figure 1, are the fruit of wheat plants in the grass (*Gramineae*) family (McCluskey 2011). Wheat kernels are also known as the caryopsis of a wheat plant. This portion of the wheat plant serves as a means to grow more wheat plants. Wheat is the second most commonly produced cereal crop in the U.S. (corn ranking first) with an average of 50 million acres planted each year (United States Department of Agriculture Economic Research Service 2017b). In the U.S., there are six classes of wheat divided by kernel hardness, color, and growth season (McCluskey 2011). These six classes include hard red spring wheat (*Triticum aestivum* L.), hard red winter wheat (*Triticum aestivum* L.), hard white wheat (*Triticum aestivum* L.), soft red winter wheat (*Triticum aestivum* L.), soft white wheat (*Triticum aestivum* L.), and durum wheat (*Triticum durum* Desf.). All classes of wheat aside from durum are hexaploid (AA, BB, and DD genomes) wheats with 42 chromosomes. Durum wheat is a tetraploid (AA and BB genomes) wheat that has 28 chromosomes. Table 1 provides an overview of some of the differences between the six classes of wheat.

Table 1. Characteristic differences between the six U.S. classes of wheat according to the U.S. Wheat Associates (2017).

Wheat class	Test weight (lb bu^{-1})	Grain moisture (%)	Protein content[a] (%)	Ash content[b] (%)	Falling number (s)
Hard red spring	61.6	12.1	14.0	1.54	381
Hard red winter	60.3	11.2	12.6	1.53	401
Hard white	63.2	9.6	12.0	1.52	400
Soft red winter	58.4	12.9	9.8	1.48	306
Soft white	60.6	9.3	10.2	1.36	336
Durum	60.4	11.5	13.6	1.59	380

[a]12 % moisture basis
[b]14 % moisture basis
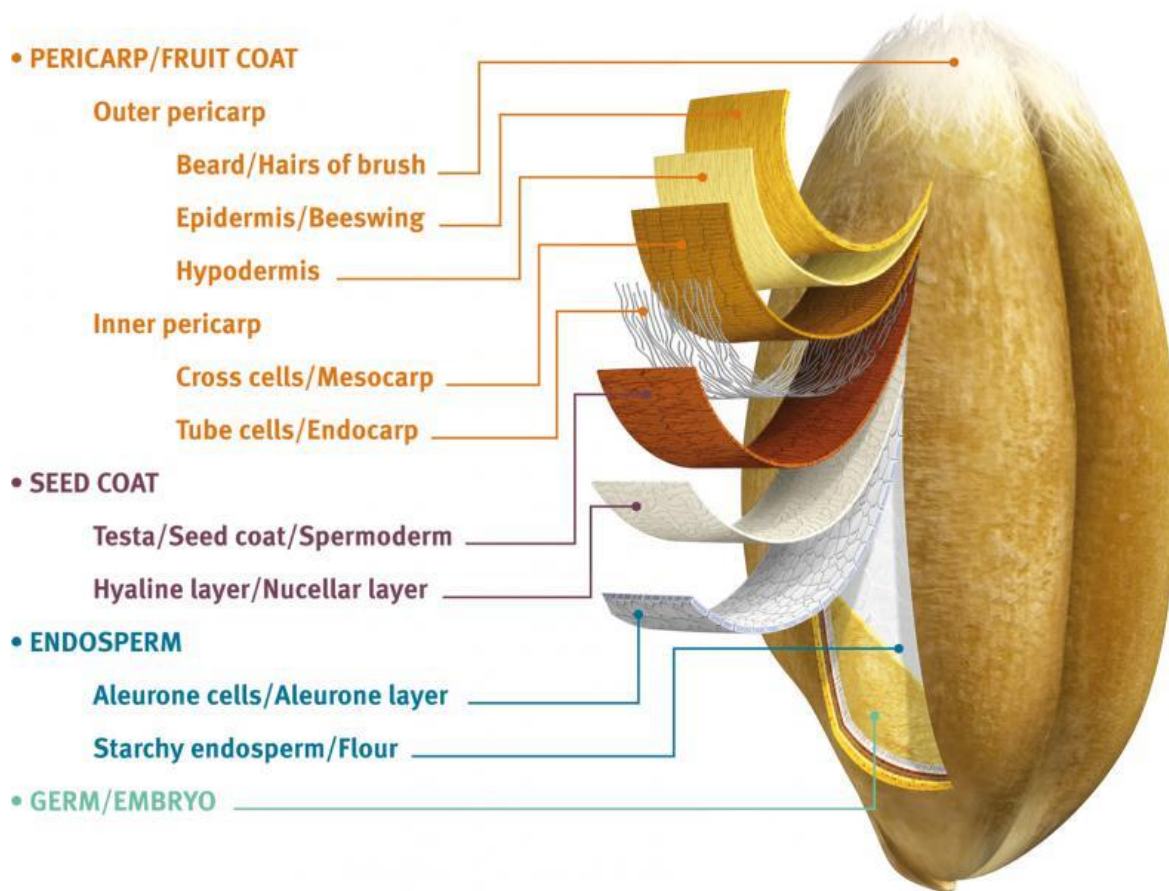
Figure 1. Cross sectional diagram of a wheat kernel. From Grain gallery (http://grain-gallery.com/en/wheat/images) by GoodMills Innovation GmbH, licensed under CC BY 2.0. Reprinted without changes from GoodMills Innovation GmbH (2015).

Hard Wheat

Hard wheat classes including hard red spring wheat, hard red winter wheat, and hard white wheat are utilized for different purposes, and are grown in different regions of the U.S. (U.S. Wheat Associates 2013). There are typically about 30 to 35 million acres of hard wheat planted per year in the U.S. (United States Department of Agriculture Economic Research Service 2017b). The average yield of hard wheat is around 40 bushels per acre, which is sold for around $4 per bushel. Hard red spring wheat is grown mainly in Minnesota, North Dakota, South Dakota, and Montana. This class of hard wheat is utilized for a variety of baked goods including hearth bread, bagels, pizza crust, rolls, and croissants. Hard red winter wheat is grown mainly in

California, Montana, North Dakota, South Dakota, Nebraska, Colorado, Kansas, and Texas (U.S.

Wheat Associates 2013). This class of hard wheat is utilized in numerous products including pan

breads, hard rolls, flat breads, Asian-style noodles, and tortillas. Hard white wheat is mainly

produced in Colorado, Kansas, and Nebraska (U.S. Wheat Associates 2013). This class of wheat

is typically utilized in Asian-style noodles, tortillas, flat bread, and pan bread. For all three

classes of hard wheat, there are numerous analyses performed to determine grain grade and

overall quality as shown in Table 2.

Table 2. Quality assessment analyses for all six U.S. classes of wheat.

	Hard red spring	Hard red winter	Hard white	Soft red winter	Soft white	Durum
Grain grade						
Test weight	X	X	X	X	X	X
Damaged kernels	X	X	X	X	X	X
Foreign material	X	X	X	X	X	X
Shrunken and broken kernels	X	X	X	X	X	X
Total defects	X	X	X	X	X	X
Wheat of other classes	X	X	X	X	X	X
Vitreous kernels	X					X
Flour/semolina quality						
Milling extraction	X	X	X	X	X	X
Moisture	X	X	X	X	X	X
Ash	X	X	X	X	X	X
Protein	X	X	X	X	X	X
Color	X	X	X	X	X	X
Gluten index	X	X	X	X	X	X
Wet gluten	X	X	X	X	X	X
Falling number	X	X	X	X	X	X
Starch pasting profile	X	X	X	X	X	
Starch damage	X	X	X	X	X	
Solvent retention capacity				X	X	
Dough quality						
Farinograph	X	X	X	X	X	
Alveograph	X	X	X	X	X	X
Extensigraph	X	X	X	X	X	
Mixograph						X
Baking/cooking quality						
Pan bread	X	X	X	X	X	
Sponge cake					X	
Cookies				X	X	
Steamed bread			X		X	
Noodles			X		X	
Pasta						X

Soft Wheat

There are two classes of soft wheat in the U.S. including soft red winter wheat and soft white wheat (U.S. Wheat Associates 2013). About eight million acres of soft wheat are planted each year in the U.S. (United States Department of Agriculture Economic Research Service 2017b). The average yield for soft wheat is around 67 bushels per acre, which is sold for around $4 per bushel. Soft red winter wheat is grown in numerous states including Missouri, Arkansas, Louisiana, Mississippi, Alabama, Illinois, Indiana, Ohio, Kentucky, Tennessee, Georgia, South Carolina, North Carolina, and Virginia. This class of soft wheat is typically utilized in products including flat bread, pretzels, pastries, crackers, and cookies. Soft white wheat is grown mainly in the North-Western U.S. including Washington, Oregon, and Idaho (U.S. Wheat Associates 2013). This class of soft wheat is often used to make Asian-style noodles, flat breads, and cakes.

Durum Wheat

Durum wheat is grown mainly in North Dakota and Montana (U.S. Wheat Associates 2013). On average, there are about two million acres of durum wheat planted every year in the U.S. (United States Department of Agriculture Economic Research Service 2017b). The typical yield of this class of wheat is about 44 bushels per acre, which is sold for an average of $6 per bushel. This class of wheat is very high in protein and has a notable amber color. In addition, the gluten strength of this class of wheat makes it ideal for making pasta. In addition to pasta, this class of wheat is utilized to make Mediterranean bread and couscous.

<center>

Escherichia coli

</center>

Escherichia coli is a bacterium that is gram negative, rod-shaped, motile, non-spore forming, facultatively anaerobic, and a member of the *Enterobacteriaceae* family (Government of Canada 2014). There are numerous serovars of *E. coli* that are not harmful and are a part of

the natural flora of the digestive systems of animals and humans. However, serovar O157:H7, which is shown in Figure 2, was identified as a pathogen in 1982 (Centers for Disease Control and Prevention 2015). Including *E. coli* O157:H7, there are over 200 serovars of *E. coli* that produce Shiga toxins, and over half of these can cause illness in humans (Sondi and Salopek-Sondi 2004; Fratamico et al. 2003). *E. coli* serovars are classified as enterotoxigenic *E. coli,* enterohemorrhagic *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli,* enteropathogenic *E. coli, or* enteroinvasive *E. coli* depending upon pathogenicity as shown in Figure 3 (Centers for Disease Control and Prevention 2015).
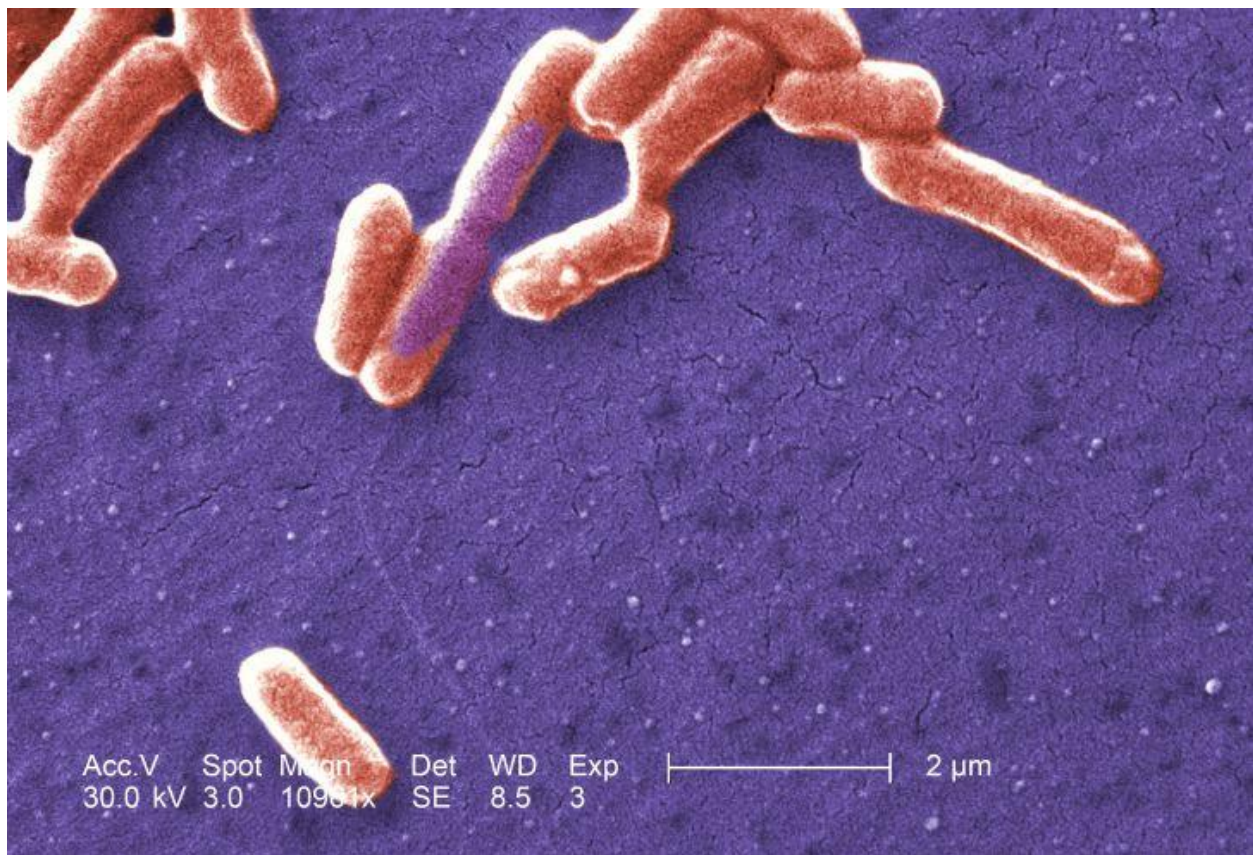
Figure 2. Image of *Escherichia coli* O157:H7. From *Escherichia coli* (https://pixnio.com/science/microscopy-images/escherichia-coli/gram-negative-escherichia-coli-bacteria-o157-h7-e-coli-o157-h7-bacterium) by Carr, J. H. Public domain. Reprinted from Carr (2017a).
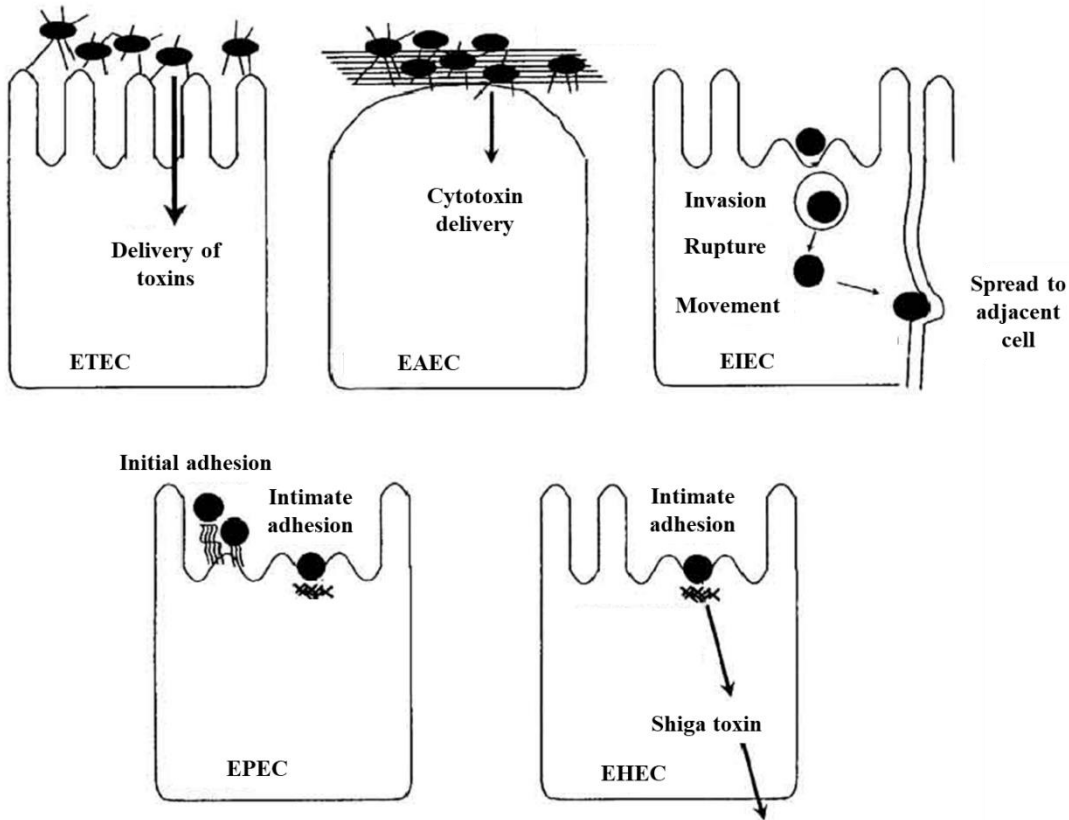
Figure 3. Six pathogenicity schemes of *Escherichia coli*. ETEC = enterotoxigenic *E. coli*, EAEC = enteroaggregative *E. coli*, EIEC = enteroinvasive *E. coli,* EPEC = enteropathogenic *E. coli*, EHEC = enterohemorrhagic *E. coli*. From *E. coli* (https://commons.wikimedia.org/wiki/File:Pathovar_Ecoli.jpg) by Nougayrede, J.-P., licensed under CC BY-SA 2.5. Reprinted with modifications from Nougayrede (2007).

Every year, about 265,000 people become ill due to *E. coli* infections in the U.S (Centers for Disease Control and Prevention 2015). *E. coli* O157:H7 is currently one of the serovars most commonly associated with illness (i.e. hemolytic uremic syndrome and hemorrhagic colitis) as it causes about 36 % of *E. coli* related illnesses in the U.S. However, non-O157 serovars including O26, O45, O103, O111, O121, and O145 are becoming an increasing cause for concern (Centers for Disease Control and Prevention 2015; Fratamico et al. 2003). *E. coli* O121 has been responsible for about 8 % of *E. coli* related illnesses, corresponding to about 3,000 cases, including cases in outbreaks due to contaminated flour (Brooks et al. 2005).

Pathogenicity of Enterohemorrhagic *Escherichia coli*

Some serovars of *E. coli* have infectious doses as low as 10 cells (Schmid-Hempel and Frank 2007). Enterohemorrhagic *E. coli* is one of the most dangerous pathotypes of *E. coli*. To cause illness, enterohemorrhagic *E. coli* must attach to the enterocytes of a host and produce Shiga toxins (Karmali 2004). These toxins are Shiga toxin type 1 (Stx1), Shiga toxin type 2 (Stx2), or variations of Stx1 and Stx2. These toxins are proteins with molecular weights around 70 kDa (Karmali 2004), and they are some of the most potent toxins produced by bacteria (Melton-Celsa 2014). Structurally, Stx1 and Stx2 are AB5 toxins with five identical B subunits and one active A subunit (made up of two parts – A1 and A2) as shown in Figure 4 (Karmali 2004). In general, Stx2 is more commonly produced by *E. coli* than Stx1.
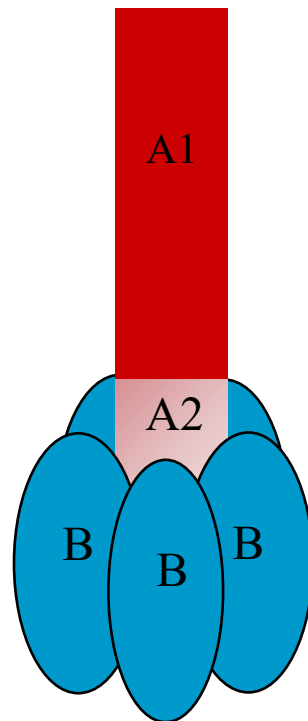
Figure 4. Pictorial representation of the structure of a Shiga toxin including the A1, A2, and B subunits. Based on Shiga toxin and its use in targeted cancer therapy and imaging by Engedal, N., Skotland, T., Torgersen, M. L., and Sandvig, K. (2011).

The spatial arrangement of the six subunits facilitates the purpose of each type of subunit (Karmali 2004). The B subunits form a donut shape with an α-helix pore in the middle. The B subunits in the pentamer serve as binding units, and they bind strongly to the globotriaosylceramide (Gb3) receptor and weakly to the globotetraosylceramide (Gb4) receptor present on human endothelial cells. There are two to three binding sites (numbered one through three) for Gb3 on every B subunit that facilitate this interaction. However, binding site number two is most commonly associated with this interaction. Despite this, binding at all three sites is required for optimal binding between Stx1 or Stx2 and a Gb3 receptor.

The A subunit, the active unit, is non-covalently bound to the B subunit pentamer (Karmali 2004). The active site of the A subunit is a glutamic acid at position 167. After the Shiga toxin has bound to and entered a target cell (via Gb3 receptors), the A subunit inhibits protein synthesis by removing an adenine from the 28S rRNA (present in the 60S ribosome) using *N*-glycosidase. The region of the A subunit that is sensitive to trypsin (amino acids 248-251) allows for cleavage of this subunit into A1 (a subunit) and A2 (a peptide) that remain connected via a disulfide bridge. The glycosidase activity remains in the A1 subunit, and the A2 peptide keeps the A1 subunit connected to the B subunits. In Stx2, the purpose of the A2 peptide also includes blocking the Gb3 binding site.

Stx1 and Stx2 are different in terms of structure as shown in Figure 5 (Melton-Celsa 2014). The B subunits of Stx1 and Stx2 differ by 2 amino acids (69 amino acids in Stx1 and 71 amino acids in Stx2), which results in different binding patterns. In addition, the A subunit of Stx1 is composed of 293 amino acids, whereas the A subunit of Stx2 has 4 additional amino acids located at its C terminus for a total of 297 amino acids.

Stx1 and Stx2 also differ in their levels of cytotoxicity (Melton-Celsa 2014). Stx2 is 100 to 400-fold more lethal than Stx1 (as determined using a mouse model where mice ingested Stx1 and Stx2). One reason for this is that the endothelial cells in the renal system are 1,000 times more sensitive to the effects of Stx2 as compared to those of Stx1. The reason(s) for this have yet to be completely defined. However, it has been demonstrated that both types of toxins have similar enzymatic activity, and both bind Gb3, but *in vitro* research has demonstrated that the binding affinities of these two toxins for Gb3 are different.
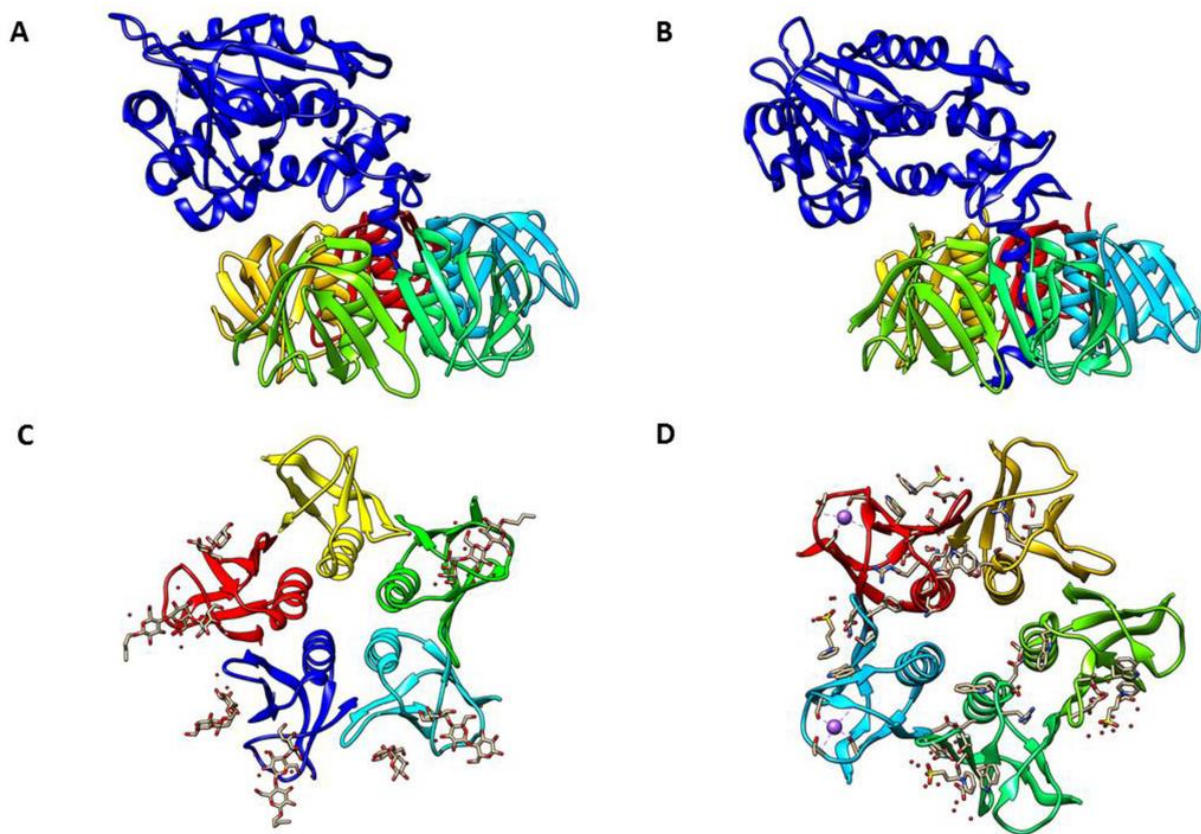
Figure 5. Shiga toxin types one and two. A: Shiga toxin type 1; B: Shiga toxin type 2; C: Shiga toxin type 1 B subunits bound to a globotriaosylceramide receptor; D: Shiga toxin type 2 B subunits bound to a globotriaosylceramide receptor. In A and B, the A subunits are dark blue, and the B subunits are shown in a variety of colors. In C and D, the B subunits are shown in a variety of colors. From Shiga toxins as multi-functional proteins: Induction of host cellular stress responses, role in pathogenesis and therapeutic applications by Lee, M.-S., Koo, S., Jeong, D. G, and Tesh, V. L., licensed under CC BY 4.0. Reprinted without changes from Lee et al. (2016).

The severity of illness caused by Shiga toxins varies depending upon the immune status of the person infected by *E. coli* and experiencing Shiga toxin intoxication. While most people experience cramps and diarrhea, some experience a much worse sequelae known as hemolytic uremic syndrome, which is potentially fatal (Melton-Celsa 2014). This condition includes the following symptoms: kidney failure, thrombocytopenia, and hemolytic anemia. The people most affected by hemolytic uremic syndrome include young children, older adults, and those with compromised immune systems (Centers for Disease Control and Prevention 2017b). There are currently no vaccines or targeted treatments available to prevent or treat Shiga toxin intoxications (Karmali 2004). However, utilization of a Gb3 receptor analogue has been suggested as it would provide another set of Shiga toxin receptors, and in doing so reduce the number of Shiga toxins bound to endothelial cells.

Presence of *Escherichia coli* in Wheat

Since *E. coli* is a zoonotic bacteria, it poses a unique set of problems in terms of the food supply (Karmali 2004). Ruminant animals including cattle and sheep carry this pathogen in their digestive system (Sargeant et al. 2007). These animals are polygastric animals, and their first stomach is called a rumen as shown in Figure 6. This stomach facilitates the fermentation of the plants they eat so they can be further digested by the other organs in the digestive system. These animals are the main reservoir of *E. coli,* even when they appear healthy (Sánchez et al. 2009). For example, Leomil et al. (2003) determined that up to 80 % of cattle are infected with *E. coli*. In addition, over 100 serotypes of enterohemorrhagic *E. coli* have been isolated from cattle (Karmali and Goglio 1994).

E. coli is shed in the fecal matter of these animals, which can contaminate food, soil, and water in multiple ways. First, when manure is used improperly as a fertilizer, it can lead to

contamination of the wheat crop it is used to fertilize (Solomon et al. 2002). However, even if the manure is handled and composted as legally required, *E. coli* may still be present. This has become a problem with increasing frequency. Second, when floods occur, irrigation water can easily become contaminated with feces directly or become contaminated through contact with water that has previously been contaminated such as surface runoff. *E. coli* can survive for long periods of time in water, so utilization of contaminated water for irrigation of cereal crops that will be used in the food chain can cause *E. coli* outbreaks.
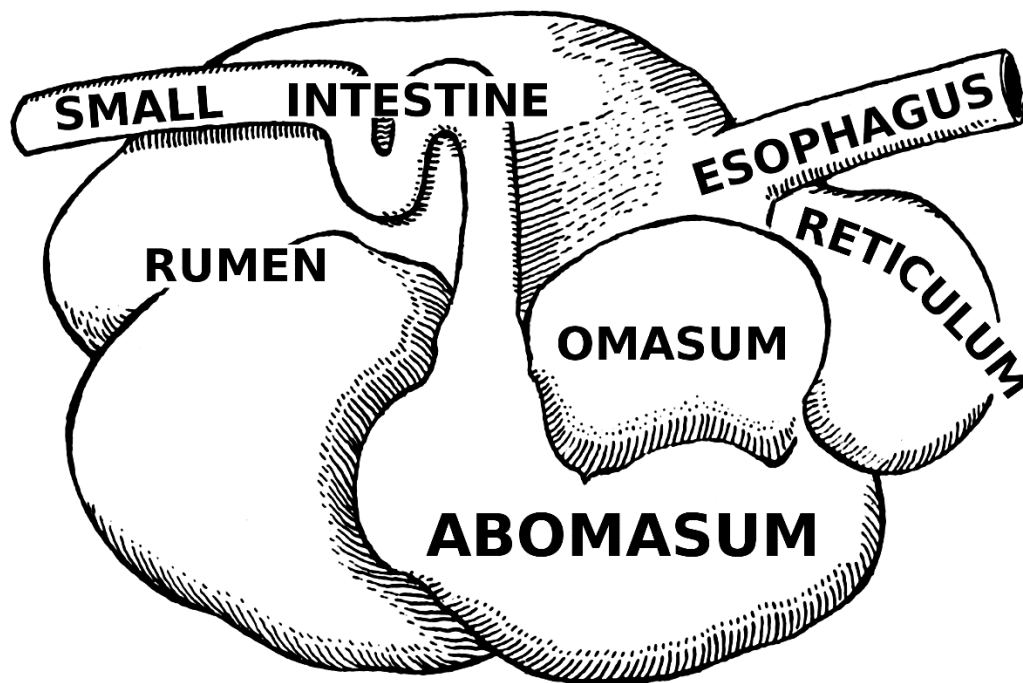
Figure 6. Polygastric digestive system with the following flow of digestion: Esophagus → rumen → reticulum → omasum → abomasum. From Abomasum (https://commons.wikimedia.org/wiki/File:Abomasum(PSF).png) by Foresman, P. S. Public domain. Reprinted from Foresman (2008).

     E. coli grows and thrives in a host such as a ruminant due to the constant warm temperature, free amino acids, and available sugars (Winfield and Groisman 2003). After being shed or otherwise removed from a host, *E. coli* faces numerous unfavorable conditions including,

but not limited to, insufficient available nutrients, variable temperature, variable pH, and osmotic stress. Exposure to these unfavorable conditions can result in the inactivation of many of the *E. coli* cells. However, those cells that do survive can become dormant (possibly viable but not culturable) and be resuscitated and multiply when conditions are favorable. While this is possible, it is unlikely that *E. coli* will survive the transition from host to secondary environment for an extended time in large numbers. However, research has shown that *E. coli* can survive in manure-amended soil for up to 231 days depending upon the temperature of the soil (Jiang et al. 2002). This can become an issue when *E. coli* is present in the soil and manure is utilized as a fertilizer, even if the manure is aged properly.

The most common serovar of *E. coli* in the food chain is O157:H7 (Andrews 2014). However, the "Big Six" non-O157 serovars including O26, O45, O103, O111, O121, and O145 can also contaminate food including wheat (Hsu et al. 2015). The prevalence of these "Big Six" serovars of *E. coli* in the food chain are as follows: 26 % (O26), 22 % (O103), 19 % (O111), 6 % (O121), 5 % (O45), and 4 % (O145) (Gould et al. 2013). The exact prevalence of these six serovars of *E. coli* in wheat has not yet been analyzed and published.

When wheat is contaminated prior to harvest, the *E. coli* is mainly present on the outside layers of the kernel including the bran and germ (Hocking 2003). This *E. coli* can contaminate milling equipment and to a lesser extent, the milled end-products. The end-products, refined flour and semolina, do not support the growth of *E. coli*, but this pathogen can survive in flour and semolina if it is present following processing. Berghofer et al. (2003) determined the frequency of *E. coli* contamination in commercial wheat samples throughout the milling process. They determined that it is most often present in tempered wheat (14 % positive) followed by wheat germ (11 % positive), bran (4 % positive), and lastly refined flour (1 % positive).

Tempering of wheat increases the moisture content and water activity of the wheat to a level that can facilitate microbial growth beyond that present on wheat prior to this treatment (Hocking 2003). In addition, milling removes the outer, more highly contaminated layers of the wheat kernel (i.e. germ and bran), which results in refined flour having a lower level of *E. coli* contamination. Research on the prevalence of *E. coli* O157 and the "Big Six" in flour is relatively sparse. However, there have been multiple recent outbreaks of *E. coli* O121 and O26 in flour from multiple milling companies in multiple countries including the U.S. and Canada (Schroder 2017; U.S. Food and Drug Administration 2016).

Other trends in the occurrence of *E. coli* in wheat have been noted in wheat. First, Richter et al. (1993) tested over 4,000 U.S. wheat samples and discovered that 12.8 % of them were positive for *E. coli*. Second, contamination of wheat with *E. coli* takes place at the slowest rate during the spring. Third, out of all six U.S. classes of wheat, durum wheat has the highest level of *E. coli* contamination (17 %), and hard red winter wheat has the lowest level of *E. coli* contamination (6.7 %).

Salmonella enterica

Salmonella spp. including *S. enterica,* shown in Figure 7, cause a foodborne illness called salmonellosis in humans (Silva et al. 2014; Jackson et al. 2013). *S. enterica* is a member of the *Enterobacteriaceae* family, gram negative, rod-shaped, facultatively anaerobic, motile, and non-spore forming. This species of *Salmonella* has over 2,600 serovars, many of which infect animals and humans. *S. enterica* is often divided by subspecies (I - enterica, II - salamae, III - arizonae, IV - diarizonae, V- houtenae, or VI - indica) or host preference (generalist, host-restricted, or host-adapted). Typically, subspecies I generalists cause salmonellosis in humans (Silva et al. 2014; Huston et al. 2002). Subspecies II-VI are typically only present in the environment and

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cold-blooded animals (Government of Canada 2011). Generalist *Salmonella* spp. are more

persistent, genetically diverse, and typically possess a plasmid coding for increased virulence

(Dandekar et al. 2014; Silva et al. 2014). After subspecies and host preference, *S. enterica* can be

further classified by serotype depending upon the antigens present on the outside of the

bacterium. These antigens include the flagellar antigen, oligosaccharide antigen, and

polysaccharide antigen.
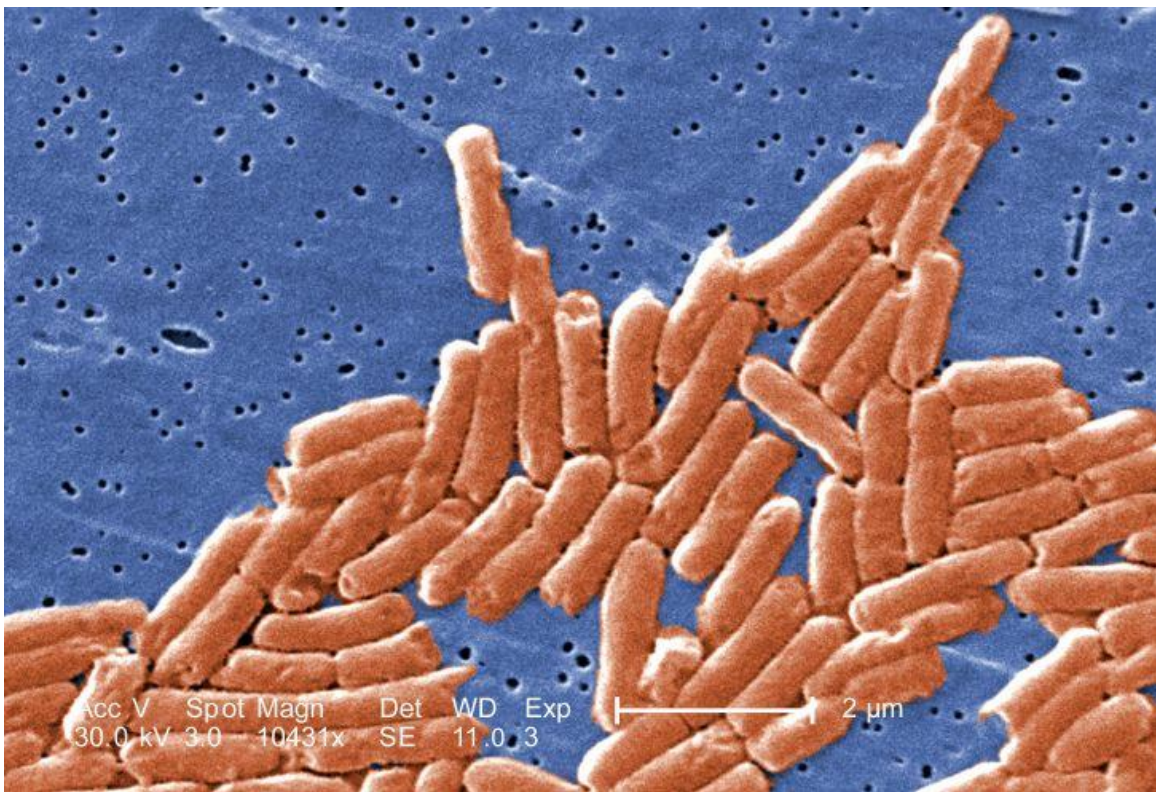
Figure 7. Image of *Salmonella enterica.* From *Salmonella*
(https://pixnio.com/science/microscopy-images/salmonellosis-salmonella/gram-negative-bacilli-
or-rod-shaped-salmonella-sp-bacteria) by Carr, J. H. Public domain. Reprinted from Carr
(2017b).

On an annual basis, *S. enterica* causes 1.2 million food-related illnesses that result in

19,000 hospitalizations and ultimately takes the lives of 450 people (Centers for Disease Control

and Prevention 2016b). The two serovars responsible for most of these cases are *S. enterica*

Typhimurium and *S. enterica* Enteritidis.

17

Pathogenicity of *Salmonella enterica*

S. enterica is present in a variety of locations and living hosts including soil, water, animals, and humans (Silva et al. 2014). The serovar most commonly associated with salmonellosis in humans due to contaminated wheat is *S. enterica* Typhimurium, which is a zoonotic generalist. However, many different serovars and strains are present in a variety of animals as shown in Table 3. Despite the numerous types of *S. enterica*, transmission pathways remain a commonality. For example, horizontal transmission of *S. enterica* from animals to humans via fecal matter is very common. In addition, it is possible for *S. enterica* to be transmitted from a mother to her offspring via vertical transmission (Hanson et al. 2016; Silva et al. 2014). *S. enterica* is also a part of the normal flora in the gastrointestinal systems of reptiles, though it is asymptomatic in this location (Clancy et al. 2016; Silva et al. 2014). Furthermore, transmission via vectors including rodents, worms, and insects is possible and allows for *S. enterica* to survive when moving between hosts (Silva et al. 2014).

Table 3. Serovars of *Salmonella enterica* present in various animals.

Animal	*S. enterica* serovars	Source
Passerine birds	Typhimurium DT40 and Typhimurium DT56v	(Silva et al. 2014; Tizard 2004)
Chickens	Typhimurium and Enteritidis	(Jackson et al. 2013)
Cattle	Typhimurium, Newport, and Dublin	(Hanson et al. 2016; Jackson et al. 2013)
Pigs	Typhimurium, Newport, Infantis, Cholerae, and Uganda	(Jackson et al. 2013; Forshell and Wierup 2006)
Sheep	Abortus	(Forshell and Wierup 2006)
Horses	Abortus	(Forshell and Wierup 2006)

Illness from *S. enterica* infections in humans is due to oral ingestion of the pathogen, which results in one of four diseases: chronic asymptomatic carriage (as previously mentioned in reptiles), enteric fever (typhoid fever), bacteremia, and enterocolitis (diarrhea) (Coburn et al. 2007). The most common diseases caused by *S. enterica* are enteric fever and enterocolitis. The three serovars of *S. enterica* that cause most enteric fever cases include Typhi, Sendai, and

Paratyphi. Bacteremia is caused largely by *S. enterica* serovars Dublin and Choleraesuis in humans. Most *S. enterica* serovars cause enterocolitis in humans. The infectious dose of *S. enterica* depends upon the serovar ingested and the strength of the immune system of the person ingesting this pathogen, but is typically 10^5 cells and 10^3 cells for typhoidal and non-typhoidal serovars, respectively (Government of Canada 2011).

Enteric fever occurs when *S. enterica* Typhi is ingested and colonizes the ileum and cecum of the small and large intestines, respectively (Coburn et al. 2007). This pathogen colonizes the intestines because it out-competes the natural flora present in these organs. Once colonized, this pathogen crosses the epithelium of the intestine through bacteria-mediated endocytosis. This takes place when this pathogen invades the M-cells of the Peyer's patches in the intestine and is taken up through dendritic cells. Following these events, *S. enterica* Typhi gains access to the circulatory system and is spread via the reticuloendothelial system. This pathogen then resides in the granulomatous foci in splenocytes and hepatocytes. *S. enterica* Typhi also survives in phagocytes, which plays a major role in its pathogenicity. This pathogen survives in cells in a *Salmonella* containing vacuole, which is a membrane-bound organelle that protects it from endosomal fusion with the oxidase complex present in phagocytes. This disease requires up to two weeks to manifest in a human following ingestion of the pathogen. The typical symptoms of this disease include fever, diarrhea, headache, nausea, and constipation.

Similar to enteric fever, enterocolitis occurs when *S. enterica* colonizes the intestines of humans (Coburn et al. 2007). The serovars that cause enterocolitis colonize the apical epithelium of the intestines. Subsequently, they cause inflammation, crypt abscesses, edema, epithelial necrosis, and fluid secretion. *S. enterica* Typhimurium causes the most severe enterocolitis in the caudal ileum, cecum, and proximal colon. This serovar recruits neutrophils within 1 hour of

infection, which is the traditional signifier of this type of disease. Then, protein-rich exudates are secreted into the lumen of the intestine by *S. enterica,* which causes irritation. This disease occurs within six to 72 hours of ingestion of *S. enterica* and causes abdominal pain and diarrhea that may or may not be bloody. Symptoms can last for up to seven days but are typically self-limiting.

Presence of *Salmonella enterica* in Wheat

The primary source of *S. enterica* is food-producing livestock, which test positive for *S. enterica* up to 90 % of the time (Forshell and Wierup 2006). However, the predominance of *S. enterica* depends upon the geographic region and the type of livestock. The main types of livestock that serve as reservoirs of this pathogen and could potentially contaminate wheat include the following: sheep, pigs, poultry, horses, and cattle. When livestock is infected with *S. enterica*, it is shed through the feces and can contaminate anything it touches. This contaminated fecal matter can contaminate wheat, soil, and water in the same ways previously discussed for the contamination of wheat, water, and soil with *E. coli*. In addition, *S. enterica* can be spread through the trading of livestock and the trade of meat that has not been heat treated.

S. enterica is essentially ubiquitous, and can survive longer outside a host than *E. coli* (Winfield and Groisman 2003). This ability to survive outside a host allows *S. enterica* to move from host to host without becoming inactive during short passages through the environment. For example, *S. enterica* present in manure is culturable for up to 21 days after being deposited onto a field as fertilizer. This pathogen can also survive in a septic system for up to 15 days. In addition, *S. enterica* can survive for an extended period in water regardless of its temperature, which could cause *S. enterica* outbreaks if the irrigation water used to water a field of wheat is contaminated. *S. enterica* can even survive when the salinity of the water increases dramatically.

When soil is contaminated with *S. enterica*, it is possible for this pathogen to survive and continue multiplying for one year, which can contaminate the crops growing in the soil. In addition, *S. enterica* has been known to persist in poultry houses for more than one year. This can result in the spread of *S. enterica* when poultry manure is utilized as fertilizer on wheat fields.

Furthermore, flying animals including flies and birds serve as vectors for *S. enterica* (Winfield and Groisman 2003). These vectors spread this pathogen very quickly throughout the environment including on fields of wheat. In addition, *S. enterica* can survive in the digestive systems of flies for the duration of their lifetime (four weeks) and infect livestock including dairy cattle and poultry. Outbreaks of salmonellosis have been linked to these vectors, which demonstrates their effectiveness at spreading *S. enterica*.

In one study by Berghofer et al. (2003), *S. enterica* was not present on any of 650 Australian wheat samples prior to milling. In addition, for these same 650 samples, no end products of milling (bran, flour, etc.) were positive for *S. enterica*. However, Richter et al. (1993) identified that 1.3 % of over 4,000 U.S. wheat samples (subsamples from all six U.S. wheat classes) tested positive for *S. enterica*. This research group also identified that *S. enterica* contamination of wheat occurs the least in the summer months. In addition, out of all six U.S. wheat classes, soft red winter wheat has the highest level of *S. enterica* contamination (2.3 %), whereas durum wheat has the lowest *S. enterica* contamination level (0.3 %).

Detection and Identification Methods

There are numerous methods in use for detecting and identifying *E. coli* and *S. enterica* that could be utilized for whole grain wheat, wheat flour, semolina, and environmental samples. The methods vary in limit of detection, cost, skill level required, and time required. Table 4

provides a general overview of these characteristics for some of the commonly utilized detection

and identification methods.

Table 4. Commonly utilized methods for the detection and identification of *Escherichia coli* and *Salmonella enterica*.

Method	Limit of detection (CFU mL^{-1})	Cost	Skill level required	Time required (days)
Traditional cultures	10	$	Low	7
Enzyme-linked immunosorbent assays	10^5	$$	Moderate	1
Pulsed-field gel electrophoresis	1	$$	Moderate	1
Polymerase chain reaction	1	$$	Moderate	1
Immunosorbent magnetic separation	1	$$	Moderate	1
Whole genome sequencing	1	$$$	High	1

Culture-Based Methods

Traditionally, the presence of *E. coli* has been determined using culture-based methods

(Fratamico et al. 2003). Biochemical identification traditionally follows culturing and can be

used to positively confirm that *E. coli* is present in a sample. There are multiple different types of

media that can be used for the culture of the *E. coli* including eosin methylene blue agar as

shown in Figure 8 (U.S. Food and Drug Administration 2017). Next, biochemical analyses

(indole test, methyl red test, Voges-Proskauer test, and citrate utilization test) are typically

performed to confirm that *E. coli* is present. For samples that are positive, serotype analysis is

typically performed. Serotyping of *E. coli* relies on two antigens on its surface (Fratamico et al.

2003). The first antigen is the O antigen, which is present on the surface polysaccharide that is a

part of the lipopolysaccharide in the cell membrane. The second antigen is the H antigen, which

is present on the protein in the flagellum. These antigens agglutinate with an antiserum to

provide the serotype of the bacterium.

Figure 8. *Escherichia coli* colonies growing on eosin methylene blue agar. From Photograph of *E. coli* colonies on EMB agar (https://commons.wikimedia.org/wiki/File:Coli_levine.JPG) by Witmadrid. Public domain. Reprinted from Witmadrid (2009).

Similarly, *S. enterica* can be detected using traditional culture methods. In fact, the U.S. Food and Drug Administration recommends that traditional culture techniques followed by biochemical tests be used for *S. enterica* detection in food processing facilities such as wheat mills (U.S. Food & Drug Administration 2007). This method includes pre-enrichment, isolation in Rappaport-Vassiliadis broth, and selective culture on xylose lysine deoxycholate agar, which is shown in Figure 9. Following these steps, the *S. enterica* is identified on the agar (morphological identification), and biochemical testing is typically performed (lactose fermentation, sucrose testing, Voges-Proskauer testing, and citrate utilization testing). In addition, if the samples are presumptively positive on the agar chosen, serological polyvalent

flagellar or Spicer-Edwards serological testing and serological somatic testing are typically

performed.

Figure 9. *Salmonella enterica* colonies growing on xylose lysine deoxycholate agar. From *Salmonella* species growing on XLD agar (https://commons.wikimedia.org/wiki/File:Salmonella_species_growing_on_XLD_agar_-_Showing_H2S_production.jpg) by Werther, licensed under CC BY 2.0. Reprinted without changes from Werther (2012).

These culture-based methods are very time consuming (five to seven days), require

trained personnel, and are not always ideal for production facilities (Abirami et al. 2016).

However, traditional culture-based methods allow for further analysis of isolates as the isolates

can be kept, which is necessary for high-quality epidemiological research (Marder 2017). Also,

culture-based methods produce fewer false-negatives, are relatively inexpensive, and can be

either quantitative or qualitative depending upon the methodology utilized (Mangal et al. 2016; Gouws et al. 1998).

Enzyme Linked Immunosorbent Assays

Enzyme-linked immunosorbent assay methods are much faster and may be easier to perform than culture-based methods (Shen et al. 2014). Most enzyme-linked immunosorbent assay methods have a limit of detection around 10^5 to 10^7 colony-forming units (CFU) mL^{-1}. This is much higher than would be ideal for detecting *E. coli* on wheat because *E. coli* has a very low infectious dose (100 cells or less). However, when enzyme-linked immunosorbent assay methods are paired with beacon gold nanoparticles and immunomagnetic nanoparticles the limit of detection is $6.8x10^2$ to $6.8x10^3$ CFU mL^{-1}. In addition, this method requires only three hours to complete.

There are five main steps to detecting *E. coli* in samples using an enzyme-linked immunosorbent assay paired with beacon gold nanoparticles and immunomagnetic nanoparticles (Shen et al. 2014). First, the immunomagnetic nanoparticles, *E. coli* antibody, and beacon gold nanoparticles are prepared. Second, the immunomagnetic nanoparticles are mixed with the sample, and the *E. coli* complexed to the nanoparticles is separated from the remaining solution using a magnetic plate. Third, the beacon gold nanoparticles are added to the complex that was separated, and excess beacon gold nanoparticles are removed. Fourth, streptavidin-horseradish peroxidase is added, and excess is magnetically removed. Fifth, the amount of *E. coli* in the sample is quantified spectrophotometrically.

These enzyme-linked immunosorbent assay methods can also be utilized for the detection of *S. enterica* (Lee et al. 1990). One enzyme-linked immunosorbent assay method available for the detection of *S. enterica* Typhimurium utilizes a monoclonal detector antibody that does not

react with other species of bacteria and only reacts with one other serovar of *S. enterica.* The limit of detection for this method is 10 cells per 25 g sample. This method requires a step of enrichment (which accounts for the very low limit of detection), but no selective culture, which cuts down the total time required for detection. The main steps in this process after enrichment of the sample include applying the sample to a microtitration plate coated in monoclonal antibodies specific for *S. enterica* Typhimurium, addition of immunoglobulin G-horseradish peroxidase, addition of the 3,3',5,5'-tetramethyl benzidine substrate solution, and analysis of the results by determining the optical density spectrophotometrically.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis is another detection and identification method, and it is utilized by PulseNet to provide scientists with a "DNA fingerprint" of a bacterium such as *E. coli* or *S. enterica* (Centers for Disease Control and Prevention 2017c; 2013). This method can be used to identify the subtype of a bacterium, but it does not always discriminate amongst closely related serotypes. Despite these things, this method is currently the gold standard for epidemiological investigations due to its ease of use and high rate of usage. However, it is slowly being replaced by methods with higher discriminatory power.

This procedure has seven main steps shown in Figure 10 that take 24 to 26 hours from start to finish. The process begins with an enrichment and selective culture. For *E. coli,* this is done by growing *E. coli* on trypticase soy agar with 5 % defibrinated sheep blood, and subsequently incubating for 14 to 18 hours. For *S. enterica,* enrichment is performed in buffered peptone water followed by selective culture on a peptone and yeast extract agar.
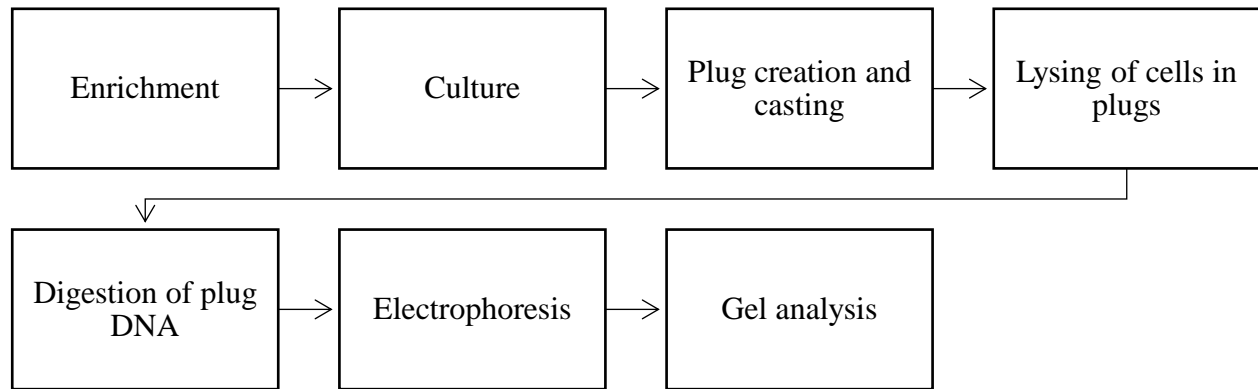
Figure 10. Outline of the steps required to perform pulsed-field gel electrophoresis according to the Centers for Disease Control and Prevention (2013).

Polymerase Chain Reaction

Polymerase chain reactions can be utilized to detect if *E. coli* or *S. enterica* is present in wheat samples. This method amplifies specific sections of DNA to detect these bacteria at low concentrations (Molina et al. 2015). There are multiple types of polymerase chain reactions, but the main four steps of these reactions include DNA extraction, denaturation of extracted DNA, annealing of specially chosen primers to sections of DNA of interest, and primer extension by DNA polymerase (McClean 1997). This process can be repeated up to 35 times in a thermal cycler to obtain larger quantities of DNA. The DNA is then analyzed using gel electrophoresis.

Multiplex polymerase chain reactions utilize sets of multiple primers to detect the presence of multiple organisms at the same time (McClean 1997). Specially designed sets of primers are utilized to do this. These primers are specifically designed so that they identify genes unique each bacterium of interest. If the primers used identify highly conserved genes, it may not be possible to discriminate between different species or serotypes of bacteria.

Real-time reverse-transcriptase polymerase chain reactions are another type of polymerase chain reactions that can be utilized to detect the presence of bacteria (Hedican et al. 2009). This method allows for both the detection and quantification of a pathogen in a short time

when coupled with either fluorescent dyes or DNA probes to provide a real-time analysis (Mangal et al. 2016). When the proper primers are utilized, this method is fast, has a detection limit of 5 CFU per 25 g sample (though this depends upon sample matrix), is quantitative, and provides more information than culture-based methods (Law et al. 2014). However, there are some draw backs to real-time reverse-transcriptase polymerase chain reactions including cost, requiring trained personnel, and false positives due to complex sample matrices (despite use of ethidium monoazide or propidium monoazide) (Bustin and Nolan 2004).

Immunosorbent Magnetic Separation

Immunosorbent magnetic separation is a detection method that utilizes specialized beads to capture bacteria (Varshney et al. 2005). These beads are immunosorbent magnetic particles that are 50 to 150 nm in diameter and have a mass of 0.5 µg. The surface of these particles is coated with antibodies that interact with the antigens present on the outside of the bacterium of interest (e.g. *E. coli*), which make them ideal for detecting specific serovars of a bacterium. The main steps in this separation technique include mixing the immunosorbent magnetic particles with the sample, separating the immunomagnetic particles that have complexed with the pathogen from the sample matrix using a magnet, washing, and enumerating the pathogen using plate culture techniques. An enrichment step may be added to the beginning of this procedure to decrease the limit of detection; however, this will increase the time required to perform the method.

This method can be utilized for detecting bacteria including *E. coli*. However, there are currently no immunosorbent magnetic particles that target the antigens present on *S. enterica,* so this method cannot be utilized for the detection of *S. enterica.* Regardless of the target pathogen, the capture efficiency of this method increases as the concentration of said pathogen decreases

(Varshney et al. 2005). Immunosorbent magnetic separation can be coupled with other detection methods including polymerase chain reactions to confirm the presence of *E. coli* colonies growing on plates. Limits of detection for this method are as low as 1 CFU per 25 g sample. However, the matrix of the sample plays a major role in the detection limit of immunosorbent magnetic separation.

Whole Genome Sequencing

Whole genome sequencing is growing in popularity as a detection and identification method for bacterial pathogens including *E. coli* and *S. enterica*. This method has higher discriminatory power than any other detection method as it provides the entire genome of a bacterium (Phillips et al. 2016). There are many steps in this detection method (especially when performed *de novo*) as shown in Figure 11, but they are divided into wet-lab preparation and genome assembly, which can be followed by population genomics if desired (Ekblom and Wolf 2014). Once the genome is completely sequenced, numerous types of information can be obtained including copy number variation, single nucleotide polymorphisms, insertion-deletions, and sequence repeats.

Whole genome sequencing is becoming increasingly popular because combining this information with epidemiological traceback information allows for a deeper understanding of foodborne outbreaks such as those due to contaminated wheat (Jackson et al. 2016). This facilitates proper clustering of cases and identification of the source of the outbreak. In addition, proper utilization of this information decreases the time required to perform the outbreak investigation, increases the number of cases linked to a source, and increases the number of clusters detected.
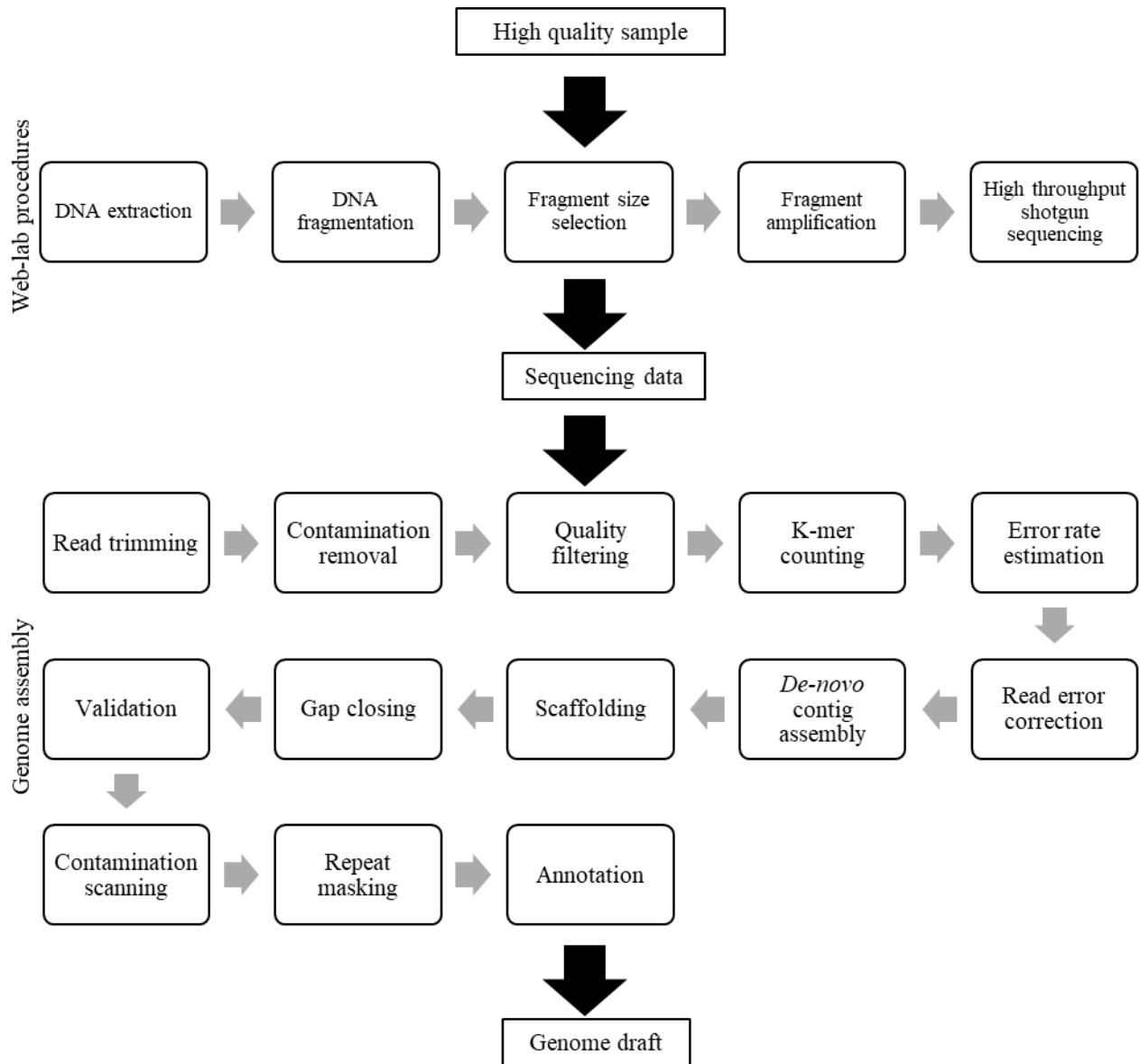
Figure 11. General overview of the steps involved in whole genome sequencing. Based on A field guide to whole-genome sequencing, assembly, and annotation by Ekblom and Wolf (2014).

Options for Preventative Controls in Flour

There are many control measures that are effective at inactivating *E. coli* and *S. enterica*, however, the best control measure must be chosen for the matrix being treated (in this case, whole grain wheat). Control measures range from using probiotics in the feed of cattle to pasteurizing wheat prior to milling as shown in Figure 12. One of the main concerns in choosing a control measure is choosing one that makes the wheat safe, but also maintains its quality. For

example, high temperatures of traditional pasteurization methods can damage the functionality of the protein and starch in grain (Shah et al. 2017). Control measures that inactivate *E. coli* and *S. enterica* in wheat are being researched with increasing frequency due to the increasing emphasis being placed upon food safety in grain. Typically, wheat is milled into flour or semolina for use in other products including bread, cookies, cakes, pasta, and noodles. These baking processes inactivate *E. coli* and *S. enterica*, greatly reducing the risk of foodborne illness. However, people often consume raw flour when they bake at home, which can result in illness.
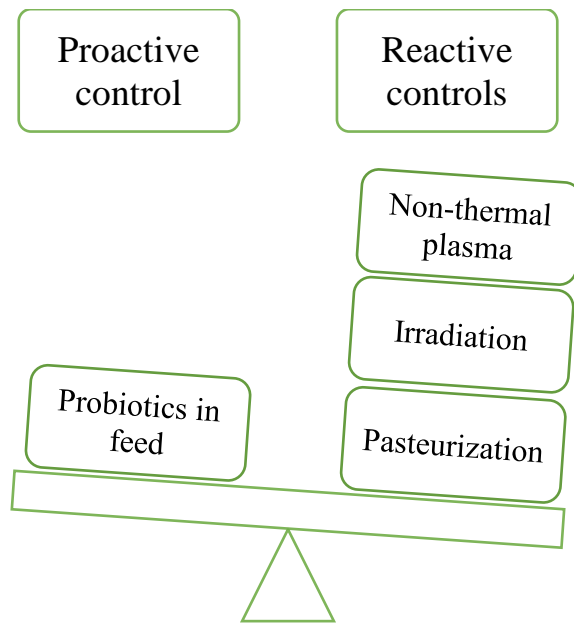
Figure 12. Measures for controlling *Escherichia coli* and *Salmonella enterica* on wheat.

Reduction of *Escherichia coli* and *Salmonella enterica* Shed in the Feces of Livestock

Reduction in the shedding of *E. coli* in the feces of ruminant animals could be utilized in the future to control the level of this pathogen in the food chain (Sargeant et al. 2007). This would minimize the amount of *E. coli* entering the environment that could end up on wheat. To achieve this, the resistance of ruminant animals to the growth of *E. coli* must be increased. Multiple methods for accomplishing this have been discussed including use of probiotics, vaccinating the animals, utilization of antimicrobials, adding sodium chlorate to an animal's

drinking water, and use of bacteriophages. The methods that show the most promise as control measures include utilization of probiotics and the addition of sodium chlorate to an animal's water. First, probiotics (in this context) are microorganisms that are fed to animals with the intent of promoting the growth of beneficial gut microbes that will make the animal healthier and inhibit pathogen growth. Multiple probiotics have been utilized on a trial basis including *Lactobacillus acidophilus* and *Propionibacterium freudenreichii.* Feeding cattle these probiotics has been shown to reduce the shedding of *E. coli* O157. It is possible that probiotics could be utilized to reduce the shedding of other serovars of *E. coli*, but this has yet to be studied and published. Second, when sodium chlorate has been added to the drinking water of cattle, it resulted in a three to four log reduction of *E. coli* in fecal shedding. Once again, only results for *E. coli* O157 were specifically analyzed and published, but it is likely that the level of other *E. coli* serovars shed by cattle were also reduced using this method. Reducing the fecal shedding of *E. coli* in cattle would reduce the risk of *E. coli* contamination of wheat when manure is spread on wheat fields.

The use of probiotics has also shown promise in reducing the fecal shedding of *S. enterica* Typhimurium in mouse models (Deriu et al. 2013). It may be possible to utilize *E. coli* Nissle 1917 as a probiotic because it competes with *S. enterica* Typhimurium for iron. Both microorganisms scavenge for iron using similar mechanisms when there are low levels of iron available, such as during the inflammatory response of a host. The initial mechanism utilized by both bacteria is the production and utilization of siderophores (chelators with a high affinity for iron) to acquire iron from the environment. However, during the inflammatory response of humans, siderophores are not efficient enough to sustain the iron requirement of these microorganisms. Due to this, these microorganisms perform salmochelin-mediated acquisition of

iron. Oral administration of non-pathogenic, commensal *E. coli* Nissle 1917 to mice reduces

fecal shedding of *S. enterica* Typhimurium. This takes place because this strain of *E. coli* out

competes this serovar of *S. enterica* for iron. In addition to inhibiting *S. enterica* Typhimurium

growth, *E. coli* Nissle 1917 also reduces the inflammation of the intestines caused by this

pathogen. Overall, utilization of this strain of *E. coli* as a probiotic appears to be a viable option

for reducing the fecal shedding of *S. enterica* Typhimurium, but more research must be

completed in other animal models to prove its efficacy. A reduction in the amount of *S. enterica*

shed in the feces of animals would result in less wheat being contaminated with *S. enterica*

provided this is the source of contamination.

Traditional Pasteurization

Traditional pasteurization involves the treatment of foods with heat for a predetermined

amount of time (Fellows 2009). Pasteurization conditions (time and temperature) are determined

by the pH of the food in conjunction with the predetermined goal of pasteurization whether that

be to inactivate an enzyme and/or inactivate a specific microbial population. This control

measure is performed as a continuous process (which is likely to be preferred in the wheat

milling industry) or as a batch process. There are multiple types of pasteurization including low

temperature long time (63 °C for 30 minutes), high temperature short time (72 °C for 15

seconds), ultra-pasteurization (138 °C for 2 seconds), and ultra-high temperature pasteurization

(138 °C for more than 2 seconds). As the temperature of the treatment increases, the time

required to inactivate *E. coli* and *S. enterica* decreases.

Pasteurization is performed as a vat process, tunnel process, or with the product in its

container (Fellows 2009). It is likely that wheat would be pasteurized in a tunnel process as this

would be the most efficient in a mill. Four types of heat exchangers utilized for traditional

pasteurization include plate heat exchangers, tube heat exchangers, triple tube heat exchangers, and tube and shell heat exchangers. Despite the effectiveness of pasteurization for inactivating pathogenic microorganisms including *E. coli* and *S. enterica*, this control measure can be detrimental to the functional, nutritional, and sensory characteristics of the material being treated. This would be detrimental to the end-use quality of wheat, so these methods may not be a viable option for inactivating *E. coli* and *S. enterica* on wheat. Traditional pasteurization methods have long been utilized as a control measure in the food industry, but they have not been utilized in the milling industry most likely due to the negative effects on wheat quality incurred from the high temperatures utilized during this control measure.

Vacuum Steam Pasteurization

Vacuum steam pasteurization has shown promise on a research scale for the inactivation of *E. coli* and *S. enterica* on low moisture foods including grain (Shah et al. 2017). This control measure utilizes decreased pressure in combination with moderate temperatures (as compared to other pasteurization methods) to inactivate pathogenic bacteria. Temperatures used for this control measure range from 60 to over 100 °C.

The main steps in this process include pre-heating (to facilitate homogenous treatment during pasteurization), pasteurization, and cooling (if needed) (Shah et al. 2017). The pasteurization step is divided into four steps: initial application of the vacuum to reduce pressure, pre-vacuum, pasteurization, and post vacuum. All steps in the process can be adjusted to suit the matrix of the material being pasteurized. One complete cycle of vacuum steam pasteurization typically takes between 20 and 25 minutes.

This method has proven effective at reducing the *E. coli* O157:H7 and *S. enterica* Enteritidis PT 30 loads on quinoa, flaxseed, and sunflower kernels (Shah et al. 2017). When

Shah et al. (2017) pasteurized these crops at 75 °C for one minute, 5.40 to 5.89 log reductions of *E. coli* O157:H7 were achieved. At these same vacuum steam pasteurization conditions, 4.01 to 5.48 log reductions of *S. enterica* Enteritidis PT 30 were attained for these crops. Due to the proven effectiveness of vacuum steam pasteurization for the inactivation of *E. coli* O157:H7 and *S. enterica* Enteritidis PT 30 on other low moisture crops, it may prove effective for the inactivation of these bacterial pathogens on wheat.

Irradiation

Irradiation is the application of radiation that can come from many sources including, but not limited to, gamma rays and electron beams (Fan et al. 2017). This irradiation causes the bonds in the DNA of bacteria to break, resulting in defects and ultimately bacterial inactivation. In this process, gamma rays are emitted from radioisotopes of multiple different elements including cesium 137 and cobalt 60; or electron beams are produced by equipment including an electron accelerator that generate high-energy electron beams. Gamma rays cannot be applied at dose rates that are as high as electron beam dose rates due to process limitations. However, gamma rays can penetrate further into grain than electron beams, making them more effective. Although, if only the surface of the grain must be treated, electron beam irradiation would be more efficient.

In the U.S., treatments of up to 8 kGy of irradiation can legally be applied to food products such as wheat (Fan et al. 2017). This method has proven effective at reducing the pathogenic load of both *E. coli* and *S. enterica* on seeds including alfalfa. The overall efficiency of this method depends upon the surface morphology of the seed being treated. For example, a treatment of 4 to 12 kGy of electron beam irradiation is unable to inactivate all *E. coli* on mung bean seeds, fenugreek seeds, and clover seeds. While there has been research on the irradiation

of wheat for many purposes, thorough research has not been published on its effectiveness for reducing pathogenic load. In addition, irradiation is currently considered a food additive, so extensive use of this control measure would need to be accompanied by consumer education to ease fears and worries.

Non-Thermal Plasma Processing

Another up-and-coming control measure for pathogens is non-thermal plasma processing (Liao et al. 2017). This method utilizes unheated plasma, which is ionized gas that contains charged particles, reactive oxygen and nitrogen species, ultraviolet photons, and excited molecules. These chemical species cause the inactivation of bacteria including *E. coli* and *S. enterica*. This preventative control is performed at low temperatures, which would maintain the functional quality, sensory characteristics, and nutritional value of the wheat being treated. Due to the this, it could possibly be applied to whole grain wheat, flour, and semolina. In addition, this method does not produce toxic byproducts and it is relatively inexpensive. However, the surface topography of the grain would play a major role in the efficiency of this control measure. For example, the crease of wheat kernels may greatly reduce its effectiveness. Further research is needed to demonstrate the effectiveness of this control measure for all six U.S. wheat classes.

<div align="center">

Recent Outbreaks Due to Contaminated Flour

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According to the Centers for Disease Control and Prevention (2011), a foodborne outbreak is "an incidence in which two or more persons experience a similar illness after ingestion of a common food, and epidemiologic analysis implicates the food as the source of the illness." There are typically multiple foodborne *E. coli* and *S. enterica* outbreaks in the U.S. every year, but not many are due to wheat (Centers for Disease Control and Prevention 2017a). In the U.S., cases associated with foodborne outbreaks are identified using the FoodNet system,

which the Centers for Disease Control and Prevention coordinates. This organization tracks

outbreaks of foodborne illness due to 10 pathogens including *E. coli* and *S. enterica*. Recent

outbreaks of these two pathogens due to wheat products are summarized in Table 5.

Table 5. Recent *Escherichia coli* and *Salmonella enterica* outbreaks in wheat products.

Bacterial pathogen	Location	Year	Wheat product	Cases
E. coli O121 and O26	U.S. (24 states)	2015	Flour	63
E. coli O121	Canada	2016	Flour	28
S. enterica Agona	U.S. (15 states)	2008	Puffed wheat cereal	28
S. enterica Typhimurium	New Zealand	2008	Flour	75

Escherichia coli

In 2015, there was a multistate outbreak of *E. coli* O121 and O26 in flour (Centers for

Disease Control and Prevention 2016a). This outbreak began on December 21, 2015 and

officially ended on September 5, 2016. In this outbreak, there were 63 cases across 24 states in

the U.S. including 17 hospitalizations and one case of hemolytic uremic syndrome. There were

no deaths associated with this outbreak. The source of this outbreak was contaminated flour

milled in Kansas City, Missouri that people consumed raw in batters and/or dough. The serovars

of *E. coli* involved in this outbreak were determined using whole genome sequencing. This

outbreak was relatively long because flour is typically bought, stored, and utilized over an

extended time period. The company responsible for milling the flour put out a series of three

recalls, each more extensive than the previous one, in accordance with all information regarding

the outbreak as it became available.

In 2016, there was another outbreak of *E. coli* O121 in flour, which took place in Canada

(Canadian Food Inspection Agency 2017). A recall of possibly contaminated flour was initially

issued on May 11, 2017, and this recall was expanded on May 26, 2017 as the outbreak

investigation progressed and more information became available. This outbreak included 28

cases across the providences in Canada including Alberta (5 cases), British Columbia (12 cases),

Newfoundland and Labrador (5 cases), Ontario (1 case), and Saskatchewan (4 cases) (Entis 2017). Fortunately, there were no deaths due to this outbreak. Throughout this investigation, it was determined that the contaminated flour was produced between October 14, 2016 and November 3, 2016. Despite this short production span, the duration of this outbreak was over six months due to the shelf life of flour. This outbreak also affected numerous other products that utilize this company's flour, which resulted in secondary recalls.

Salmonella enterica

There have been very few multistate outbreaks of *S. enterica* in wheat products in the U.S., but there was an outbreak of *S. enterica* Agona in puffed wheat cereal in 2008 (Centers for Disease Control and Prevention 2008). This outbreak included 28 cases across 15 states. This outbreak did not include any deaths, but it did include eight hospitalizations. The onset of illness for these 28 cases of salmonellosis began on January 1, 2008 and proceeded through April 10, 2008. The contaminated cereal was produced at a facility in Minnesota. The presence of *S. enterica* Agona was detected through routine testing at the cereal processing company, which resulted in a recall of the product. Pulsed-field gel electrophoresis was utilized to create the outbreak cluster as all cases involved had indistinguishable pulsed-field gel electrophoresis patterns that were identical to those of the isolates from the Minnesota processing facility.

In addition, there was an outbreak of *S. enterica* Typhimurium in wheat flour in New Zealand in 2008 (McCallum et al. 2013). This outbreak was due to the consumption of raw wheat flour. Once again, pulsed-field gel electrophoresis was utilized to identify the outbreak cluster after indistinguishable pulsed-field gel electrophoresis patterns were identified in isolates from unopened bags of flour and isolates from the ill humans. Throughout this outbreak, there were 75 cases identified that included 12 hospitalizations and no deaths. The onset of illness for

these cases ranged from October 13, 2008 to January 28, 2009. Cases were reportedly ill for anywhere between two and 21 days. The contaminated flour was produced between September and October 2008. All flour produced during these dates was recalled in response to the outbreak investigation.

Hazard Analysis and Critical Control Point

Hazard Analysis and Critical Control Point (HACCP) plans are utilized in the food industry to prevent foodborne illness and injuries (U.S. Food & Drug Administration 2017). These plans are coupled with other practices including good agricultural practices, good manufacturing practices, and standard operating procedures. The combination of these preventative measures reduces the risk of illness and/or injury due to consuming unsafe food. Food, including wheat flour and semolina, can be unsafe due to physical, chemical, and/or biological hazards. HACCP plans seek out and mitigate the risks associated with all three types of hazards. There are seven main principles involved in the implementation and maintenance of HACCP plans including the following: hazard analysis, critical control point identification, establishment of critical limits, monitoring of critical control points and their associated critical limits, establishment of corrective actions, establishment of verification procedures, and the maintenance of all records associated with a HACCP plan. The development of a HACCP plan involves representatives from all areas involved in the manufacturing of a product. In addition, HACCP plans incorporate numerous types of information relevant to the product from its origin to the time of its consumption (i.e. farm to fork). While not all this information will be directly written in the HACCP plan, it may be a part of a risk analysis that demonstrated the need for hazard control in the form of a HACCP plan.

Although HACCP plans are relatively new to the wheat milling industry, they are being implemented with increasing frequency. One HACCP plan that is readily available is a Generic HACCP Plan for Millers of Grains, oilseeds & Pulses developed by the Canadian Grain Commission (2017). This HACCP plan begins with a finished product description that very specifically outlines the characteristics of grain and its uses, which provides necessary background information for identifying and controlling all hazards. In addition, this HACCP plan outlines the process through which grain goes through prior to being sold. Outlining this entire process is vital to identifying the steps where hazards may be controlled. In this plan, numerous types of physical, chemical, and biological hazards are identified and addressed. Some examples include pathogenic bacteria on packaging materials, nails and wood slivers on pallets, pathogenic bacteria and mold on the grain, pesticide residues on the grain, physical hazards (e.g. glass, metal, and rocks) in the grain, and pathogenic bacteria in water utilized during the milling process. After identifying all hazards, the plan goes step-by-step through the mitigation of each hazard. While HACCP plans must be tailored for each grain processing facility, this plan provides a good representation of what a HACCP plan should include.

Mitigating Foodborne Illness in the Home

Though HACCP plans are a commonplace for mitigating the risk of foodborne illness in food production facilities, there are often few guides developed specifically for consumers to utilize in their homes for specific products. One exception to this is that the Home Baking Association developed a guide for baking safely at home. This guide is called Baking Food Safely 101 (Home Baking Association 2018), and is readily available online. In this guide, there are six simple safety steps provided along with an easy to follow checklist that empowers home bakers to bake safely. These six simple steps are outlined in Figure 13 below. In addition, this

guide also provides a list of temperatures that various baked goods should reach while baking. For example, cheesecakes should be baked to 150 °F, stuffing and casseroles should be baked to 165 °F, custard pies should be baked to 175 °F, yeast breads should be baked to 200 °F, and cakes should be baked to 210 °F. While this guide is not as comprehensive as a HACCP plan, it does provide consumers with things they can do to minimize their risk of becoming ill while baking at home.

```
┌─────────────────────┐     ┌─────────────────────┐     ┌─────────────────────┐
│ Store raw ingredients│    │ Prepare for baking  │     │ Keep raw ingredients │
│ away from ready-to-  │──> │ • Tie back long hair│──>  │ and batter separate  │
│ eat food            │     │   and wear an apron │     │ from baked goods     │
│                     │     │ • Clean counters and│     │                      │
│                     │     │   all utensils/pans │     │                      │
└─────────────────────┘     └─────────────────────┘     └─────────────────────┘

┌─────────────────────┐     ┌─────────────────────┐     ┌─────────────────────┐
│ Bake to the proper  │     │ Clean all utensils/ │     │                      │
│ temperature         │──>  │ pans and surfaces   │──>  │ Wash hands           │
│                     │     │ with hot, soapy     │     │                      │
│                     │     │ water               │     │                      │
└─────────────────────┘     └─────────────────────┘     └─────────────────────┘
```
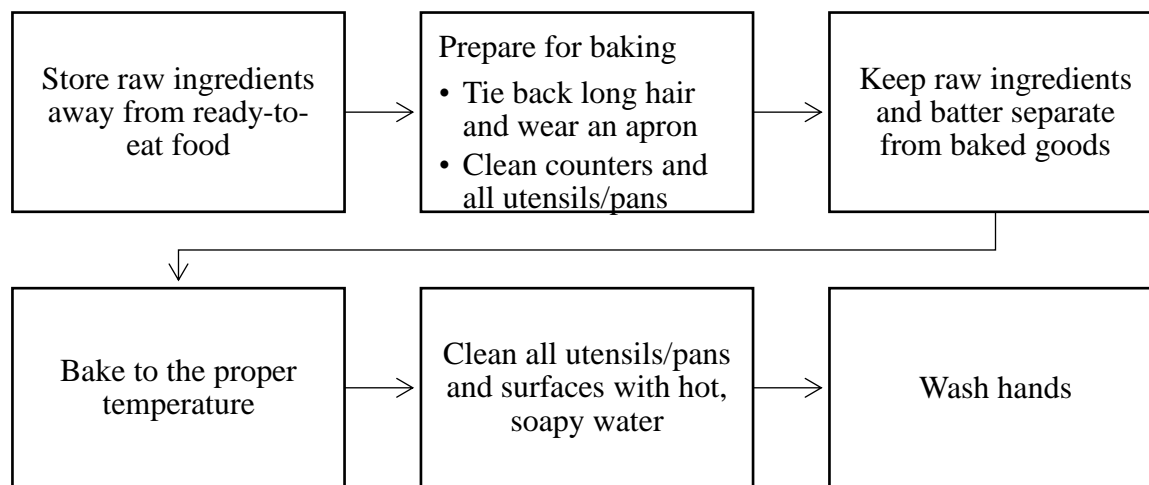
Figure 13. Six simple steps for baking safety at home according to the Home Baking Association (2018).

Economic Burden of Foodborne Illness

The burden of foodborne illness reaches far and wide, which is clearly demonstrated in the economic burden of these events. The annual cost of foodborne illness due to 15 specific pathogens including *E. coli* O157:H7, non-O157 *E. coli,* and *S. enterica* is estimated by the United States Department of Agriculture Economic Research Service (United States Department of Agriculture Economic Research Service 2017a). The economic burden of these pathogens is analyzed for these specific pathogens because they account for over 95 % of foodborne illness and associated deaths in the U.S. These cost estimates account for medical costs, productivity

loss, premature death, and the willingness of people to pay for a reduced risk of mortality. These costs are predominately paid by consumers when foodborne illness occurs.

The outcomes of foodborne illness of *E. coli* O157 and non-O157 serovars include the following: did not visit a physician and recovered, visited a physician and recovered, hospitalized, hospitalized and recovered without hemolytic uremic syndrome, hospitalized with hemolytic uremic syndrome and recovered, hospitalized with both hemolytic uremic syndrome and end stage renal disease but recovered and later died prematurely, hospitalized and died but did not have hemolytic uremic syndrome, and hospitalized and died from hemolytic uremic syndrome (United States Department of Agriculture Economic Research Service 2017a). The estimate for the annual cost of foodborne illness due to *E. coli* O157:H7 was $271,418,609 in 2013 (an updated value is not available). This included a total of 63,153 cases including 2,138 hospitalizations and 20 premature deaths. As the outcome of these cases increased in severity, the estimated case cost increased. For example, the estimated cost of a case that recovered without visiting a physician was $31.80, but the cost of a case that was hospitalized and died from hemolytic uremic syndrome was $8,713,339.69. The costs associated with non-O157 *E. coli* cases have been less despite a higher total case count. The estimate for the total annual cost due to these pathogens in 2013 was $27,364,561 (an updated value is not available). This included a total of 112,752 cases including 271 hospitalizations and 1 premature death. Once again, as the outcome of these cases became more severe, the estimated case cost increased. For example, the estimated cost of a case that recovered without visiting a physician was $31.80, but the cost of a case that was hospitalized with both hemolytic uremic syndrome and end stage renal disease but recovered and later died prematurely was $6,807,151.81.

There are four outcomes for foodborne illness due to nontyphoidal salmonellosis including the following: did not visit a physician and recovered, visited a physician and recovered, hospitalized, hospitalized and recovered, and hospitalized but died (United States Department of Agriculture Economic Research Service 2017a). The overall cost estimate of foodborne nontyphoidal salmonellosis was $3,666,600,031 in 2013 (an updated value is not available). This cost was due to a total of 1,027,561 cases including 19,336 hospitalizations and 378 premature deaths. In 2013, the estimated economic burden of a case that recovered but did not visit a physician was $56.53, but the economic burden of a case that was hospitalized and died was $8,657,357.03.

In addition to the costs that consumers pay, firms producing wheat and other food products also incur costs related to foodborne illness (Golan et al. 2000). First, implementation costs including the development of food safety plans (e.g. HACCP plans) and training of employees are paid by firms. These costs are paid initially to set up food safety methods. Second, operation costs related to foodborne illness mitigation are also paid by firms and include the cost of equipment, record keeping, food safety system(s), and microbiological testing. These costs are paid regardless of if foodborne illness occurs, but they could increase if consumers become ill. Third, firms also pay prevention costs – costs associated with foodborne illness mitigation strategies, product liability, failure costs due to recalls, logistic expenses, and losses in brand equity. Ideally, firms would incur minimal prevention costs if all HACCP plans and other food safety systems (if applicable) are implemented and maintained properly. However, this is rarely the case as no food processing facility or process is perfect. Even though recalls happen, it is possible to minimize costs by acting quickly to minimize risk of illness and maintain consumer

loyalty. Overall, there are many elements that determine the cost of foodborne illness incurred by a firm.

There are also costs of food safety that the public health sector is responsible for. These include, but are not limited to, disease surveillance, outbreak response, regulation, and consumer information costs (Scharff 2015). If it were not for research on foodborne illness mitigation, quick responses, and surveillance, there would likely be a much greater cost of foodborne illness due to an increased rate of illness. These costs are both proactive and reactive in the case of foodborne illness. As such, these costs can increase as the number of cases of foodborne illness increase. However, as with HACCP system implementation, it is likely that money spent on the proactive costs in this sector would reduce the reactive costs.

OVERALL SUMMARY AND FUTURE RESEARCH

Summary

The six classes of wheat grown in the U.S. are all different in terms of composition and end-uses. In addition, they are all grown in different areas of the U.S. and have a variety of associated quality analyses. Despite all these differences, they are all at risk for both *E. coli* and *S. enterica* contamination. Wheat can easily be contaminated by these pathogenic bacteria because it is grown outside. *E. coli* and *S. enterica* are present in animal manure, soil, and water; all of which can easily contaminate wheat. Due to these factors, about 1% of wheat is contaminated with *E. coli* and/or *S. enterica*.

While the infectious dose of *E. coli* is much lower than that of *S. enterica*, both pose a threat to human health. *E. coli* produces one of the most dangerous bacterial toxins, known as Shiga toxin, when inside a host. This toxin inhibits protein synthesis, which causes cell death and numerous symptoms from diarrhea to kidney failure. *S. enterica* does not produce a toxin but invades the endothelial cells of humans most often causing enterocolitis. Despite these differences between *E. coli* and *S. enterica*, both are dangerous to human health and must be controlled in the wheat milling industry to decrease the rate of foodborne illness due to contaminated wheat products.

Due to the uses of wheat flour and semolina, control measures utilized to mitigate the risk of *E. coli* and *S. enterica* on wheat must inactivate these bacterial pathogens without damaging the functional polymers present in the wheat kernels. Regardless of the control measure, it is likely that a very low temperature will need to be coupled with a long treatment time to meet these requirements. Low temperature pasteurization treatments, irradiation, and non-thermal

plasma processing could be options for controlling *E. coli* and *S. enterica* on wheat. However, more research must be performed before their implementation.

Future Research

There is considerable introductory research that has been successfully published on wheat, *E. coli*, *S. enterica*, methods of detection and identification of bacterial pathogens, and methods of inactivating bacteria. Despite this, there is much to be done to decrease the risk of illness associated with consuming wheat products.

To begin, it is vital to continue research on the abundance of *E. coli* and *S. enterica* on wheat. This research should include not only the prevalence of these bacteria on wheat, but also the specific strains present. It would also be beneficial to determine if there are geographic trends in the prevalence of these bacterial pathogens on wheat. This information would provide the basis for directing control methods, so they can be as effective as possible.

Next, research must be done to assess the efficacy of probiotics, pasteurization, irradiation, and non-thermal plasma treatments for the prevention and inactivation of *E. coli* and *S. enterica* on wheat. This research should include optimization of these processes for each class of wheat (if it varies with class) and pathogen so that the transition to industry is as seamless as possible.

Lastly, it would be greatly beneficial to determine the amount of money saved and overall decrease in *E. coli* and *S. enterica* outbreaks in wheat products due to the implementation of these control measures. This would provide a way to measure the success of these control measures and provide direction for further research by possible identifying areas that still need to be addressed.

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