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Heat sensitization of human enteric viruses by plant extracts and validation of their thermal inactivation by bacterial surrogates

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ABSTRACT

Human enteric viruses including hepatitis A virus (HAV) and human noroviruses (HNoV) predominantly are linked to foodborne outbreaks worldwide. Heat has been historically used to inactivate foodborne pathogens and spoilage microorganisms to increase safety and also prolong shelf-life. Recent foodborne viral outbreaks have enhanced the need for research to determine optimal processing approaches and thermal inactivation parameters such as D- and z-values, for their control. Heat-sensitization of foodborne viruses by "generally recognized as safe''(GRAS) substances can potentially reduce their time-temperature inactivation parameters during processing. HAV reportedly has higher resistance to heat (in buffer the D72°C was 0.9 min) than known vegetative bacteria. Therefore, non-pathogenic surrogates with higher thermal resistance are required for process validation. This present study determined and compared the D- and z-values of HAV (~7 log PFU/ml) and Tulane virus (TV; ~6 log PFU/ml), a cultivable HNoV surrogate, with curcumin (0.015 mg/ml), gingerol (0.1 mg/ml), or grapeseed extract (GSE; 1 mg/ml) at 52 to 68 ^{o}C in 2-ml glass vials. Decreased D-values (52 to 58 ^{o}C) for TV with curcumin from 4.32±0.25 to 0.62±0.17 min, gingerol from 4.09±0.18 to 0.72±0.09 min and GSE from 3.82±0.18 to 0.80±0.07 min, from initial 4.59±0.28 to 1.08±0.16 min in buffer wre observed by both linear and Weibull models, with similar trends for HAV (56 to 68 ^{o}C). This study also determined the D- and z-values of *Staphylococcus carnosus* strains (CS-299 and CS-300) as potential HAV surrogates under modified growth conditions (higher temperature, salt and low pH). D-values (by the linear model) of CS 300 grown at 37 ^{o}C at 56 to 68 ^{o}C in 2-mL glass vials were 6.18±0.25 to 0.54±0.12 min and increased to 7.09±0.15 to 0.59±0.06 min when grown at 42 ^{o}C, in 4% NaCl, pH 6 and further increased as a lawn. A similar trend was observed

for CS 299. Overall, this research determined the decreased time-temperature parameters needed to inactivate enteric viral surrogates and the increased heat resistance of bacterial surrogates for application in process validation for the thermal inactivation of HAV.

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CHAPTER I

LITERATURE REVIEW

1. Foodborne viruses:

1.1 Hepatitis A virus (HAV):

Hepatitis A virus is classified within a unique genus *Hepatovirus*, of the *Piconaviridae* family that is a non-enveloped, 30 nM in size, small virus (Zell et al., 2016). The positive sense single-stranded HAV RNA genome has similar molecular organization and replication mechanisms to other Picornaviruses (Beard et al., 2001). The length of the HAV RNA genome is about 7.5 kb, with an untranslated nucleic acid segment covalently linked to a small virally encoded protein (Lemon et al., 2018). The single open reading frame of HAV RNA codes for a giant polyprotein, whose initiation is under the control of a highly structured internal ribosome entry site (IRES) within the ORF (Lemon et al., 2018; Yang et al., 2008). Similar to other positive-single-stranded RNA viruses, the RNA genome is infectious that generates virus after host cell transfection (Lemon et al., 2018). The single ORF of HAV comprises of three distinct sections namely- P1, P2, and P3, where P1 encodes for the 3 main viral capsid proteins,VP1, VP2, and VP3 whereas, P2 and P3 regions code for non-structural proteins which are required for formation of virions and replication of RNA (Nainan et al., 2006; Jeong et al, 2010; Bozkurt et al., 2015). HAV may be classified into seven genogroups based on sequences of capsid genes as "GI to GVII, where only GI, GII, GIII, and GVII, are found associated with humans, and GIV, GV, and GVI strains are found in simians" (Robertson et al., 1992; Bozkurt et al., 2015). Among HAV genotypes, GI is the most prevalent with sub-genotypes that include 80% of human isolates/strains (Kokkinos et al., 2008; Bozkurt et al., 2015). Hepatitis A virus (HAV) is reported to have an incubation period of 14 to 28 days, and causes liver disease with symptoms that can be occasionally quite severe, including fever, malaise, loss of appetite, abdominal discomfort, diarrhoea, nausea, dark-

coloured urine, yellowing of skin and jaundice that can last for few weeks up to six months (Lemon et al., 2018). This disease usually affects children, old people and immunocompromised individuals, while asymptomatic individuals can also transmit HAV. Around 2,000 cases of HAV illnesses are reported in the US each year (CDC, 2018). Hepatitis A virus is spread through the fecal-oral transmission route by contaminated water, food, fomites or poor personal hygiene of infected persons (WHO, 2018). HAV can withstand mild food-processing techniques that can typically inactivate bacterial pathogens (WHO, 2018). Outbreaks of Hepatitis A since past ten years in the US have been summarized in Table 1.1. Previous studies report that HAV is a potent virus having high resistance to commonly used inactivation treatments (Calci et al., 2005; Bozkurt et al., 2014; Bozkurt et al., 2015; Lemon et al., 2018). However as prophylactic and preventive measures against HAV, currently there are three commercial vaccines available in the United States, namely Havrix® and Vaqta® (against HAV alone) and Twinrix® that works against both HAV and hepatitis B (HAB) (CDC, 2018; ANON, 2006). Despite the availability of these vaccines that can be cost-prohibitive and expensive for some groups, the incidences of HAV has been on the increase globally and in the US due to travel, trade and also unhygienic conditions in some cases. One such example is the HAV outbreak in 2018 which affected California and some states of mid-west that resulted in many hospitalizations. This was due to drug use and improper sanitation conditions among the homeless people in the region (CDC, 2018). However, the foodborne outbreaks are also increasing and so measures to control and inactivate this virus in food systems have been researched (as outlined in the inactivation strategies sections below). Validation of the inactivation parameters in processing also needs to be undertaken.

1.2 Human Norovirus (HNoV):

Human noroviruses (HNoV) are the primary agents that cause acute human viral gastroenteritis that affects all ages and HNoV illnesses and continue to be prevalent worldwide. They are responsible for "1 illness in every 15 infected people", with about thousands of hospitalizations and 800 deaths each year resulted from the consumption of contaminated fresh fruits and vegetables, marginally processed foods, shellfish, ready-to-eat items including sandwiches and bakery items, as well as water and ice (Hall et al., 2013). Approximately 1 out of every 5 cases of acute human gastroenteritis globally that leads to diarrhoea and vomiting, with 685 million cases are associated with HNoV (CDC, 2018). Once infected with HNoV, after an incubation period of typically 24 to 72 h, a person may suffer from vomiting, non-bloody diarrhoea, abdominal cramps and nausea, which are typical symptoms of acute gastroenteritis. HNoV illness can be serious in children, old people and immune-compromised individuals which can lead to hospitalization and death (Atmar et al., 2006). Emerging strains are becoming more virulent. Symptoms in otherwise healthy individuals last for about 48 to 72 h and is a self-limiting disease.

HNoV was first identified in 1973 by Kapikian using immune electron microscopy from infectious stool samples derived from a human gastroenteritis outbreak in Norwalk, Ohio, therefore, it is termed Norwalk virus (the prototype human norovirus GI) (Kapikian et al., 1973). These viruses are implicated in various outbreaks in nursing homes, cruise ships and hospitals. HNoV of the *Caliciviridae* family, comprise of non-enveloped, small (30 nM), positive-sense, stranded 7.5 kb RNA, which is covalently attached to viral protein genome (Zheng et al., 2015). HNoV have three open reading frames, known as ORFs, which encode eight viral proteins (Bull

et al., 2006). ORF-1 encodes a polyprotein which further comprises of RNA-dependent RNA polymerase (RdRp). six non-structural proteins and viral protease and (Zheng et al., 2015). Structural proteins namely viral protein 1 (VP1) and viral protein 2 (VP2) are encoded by ORF-2 and ORF-3, which give icosahedral symmetry of the capsid and creates a hollow or cup-like structure on the surface of the virus (Bull et al., 2006). HNoV interacts with histo blood group antigens (HBGAs) which are specific carbohydrates present on cell surface of the host (Zheng et al., 2015). Noroviruses have five genogroups depending on the sequence of the capsid and RdRp regions, as "GI (Norwalk virus), GII (Snow Mountain virus), GIII (bovine enteric calicivirus), GIV (Alphatron and Ft. Lauderdale viruses), and GV (murine norovirus) and only GI, GII, and GIV infect humans" (Zhen et al., 2015). Currently, there are 9 genotypes of GI and 22 genotypes of GII which have been identified, and over 30 genotypes of HNoV have been discovered till date (Karangwa et al., 2017; Chen et al., 2018). Of these, GII.4 was known as the most prevalent genotype and responsible for approximately 60% of HNoV outbreaks. HNoV GI is more resistance to inactivation and is associated with a high number of berry-related and shellfish-related outbreaks (Parra et al., 2017). HNoV outbreaks in the US are summarized in Table 1.2.

Commercial vaccines are currently unavailable against HNoV. Until recently, most HNoV genogroups were not cultivable in the lab. However, recently, only some genogroups (GI and GII) have been cultivated in the lab using human intestinal enteroids and bile was found to be a critical factor required for HNoV replication in enteroids (Etteyabi et al., 2016). However, due to lack of expression of histo blood group antigens in intestinal cells, HNoV were rapidly inactivated by treatments (Etteyabi et al., 2016). Yet, research is still on-going to get reproducible titers at high enough levels to determine inactivation and detection mechanisms.

Therefore, most studies rely on the use of cultivable surrogates to determine inactivation approaches to control and prevent the spread of HNoV (Bozkurt et al., 2015; Etteyabi et al., 2016).

2. Need for surrogates:

Surrogate microorganisms are harmless to general human population having comparable resistance traits to pathogenic or spoilage organisms (Hu et al., 2017). They can be used as alternates for food process trial testing or determining effects of preservatives on a food (Hu et al., 2017). Validation of a process depends on its consistency to produce required results under predetermined specifications (FDA, 2014). An ideal experiment would involve intentional contamination with the hazards followed by consistent reduction of the foodborne pathogens throughout the process (FDA, 2014). However, using target pathogen could lead to possible contamination and outbreaks. Therefore, a surrogate organism could be used which mimics thermal resistance and survival profile of target pathogen for process validation (Mossel, 1995; FDA 2014; Hu et al., 2017).

Other important traits for surrogates includes ease of cultivability of organisms, that can generate large data sets (Sinclar et al., 2012). Therefore, criteria of selecting an appropriate surrogate for a target pathogen largely depends on these characteristics of surrogates including non-pathogenicity, not causing food spoilage, similar inactivation characteristics, resistance profiles, transmission route and attachment characteristics as the target pathogen or that can be used for prediction of behavior of target pathogen, demonstrates stable and consistent growth kinetics, ease of enumeration, rapid detection, easily differentiated, genetically stable and provides reproducible results (Busta et al., 2003; Hu et al., 2017). An example includes use of

Escherichia coli O157:H7 strain ATCC 700728, to study the survival of pathogenic *E. coli* O157:H7 in field-inoculated lettuce (Moyne et al., 2011). Another is the use of *Salmonella enterica* serovar Typhimurium 3985, a vaccine candidate strain, to determine *Salmonella* persistence in soil and vegetables (produce) by contaminated compost and irrigation water use (Islam et al., 2004). Others include *Listeria innocua* strain CIP 80–12 and *Clostridium sporogenes* strain CIP 79–3 as potential surrogates of *L. monocytogenes* and *C. botulinum*, respectively, for monitoring their growth and characteristics during field production of parsley (Girardin et al., 2005).

In the case of HNoV, *in vitro* cultivation models for propagation (Jones et al., 2014; Ettayebi et al., 2016) though reported, their availability and routine propagation for use in viral inactivation continues to be limited. Therefore, several cultivable surrogates have been used to determine inactivation by physical and chemical processes for including feline calicivirus (FCV-F9), murine norovirus (MNV-1), Tulane virus (TV), porcine sapovirus (PoV), and also bacteriophages (Hirneisen et al., 2009; Kamarasu et al., 2018; Joshi et al., 2019; Ailavadi et al., 2019; Bozkurt et al., 2015; Lacombe et al., 2018).

3.Surrogates for HNoV:

3.1 Feline Calicivirus (FCV):

Feline calicivirus (FCV) that belongs to the genus *Vesivirus* in the same *Caliciviridae* family as HNoV is known to cause respiratory diseases in cats (Carter et al.,1992; Stuart and Brown, 2007). FCV is a 27-40 nm in size, icosahedral in shape and non-enveloped with a positive-sense, single stranded 7.7 kb RNA (Carter et al., 1992). It is cultivable in laboratory using the host Crandell-Reese feline kidney (CRFK)cell line (Carter et al., 1992). FCV binds to α-2,6-sialic

acid receptors (Stuart and Brown, 2007; Radford et al., 2007). Thermal inactivation studies of FCV in cell culture media and food matrices are described in Table 1.4. FCV-F9 (7 log PFU/ml) showed ~1.5 log PFU/ml reduction with 70% ethanol for 1 min and to non-detectable levels upon treatment with 100 mM citric acid (pH 2) and 100 mM carbonate buffer (pH 10) for 30 min at 37 °C and 5.3 log PFU/ml reduction using commercial bleach at 1,000 ppm chlorine at room temperature for 5 min on stainless steel discs (Cannon et al., 2006; Cromeans et al., 2014). FCV-F9 was decreased to levels that were not detectable from initial titers of 7 log PFU/ml using 5% trisodium phosphate for 30 s at room temperature (Su and D'Souza, 2011). These reports indicate that FCV may not be an ideal HNoV surrogate since it has a different transmission route, unlike HNoV and lower resistance to elevated temperatures and chemical treatments.

3.2 Murine Norovirus (MNV-1):

Murine norovirus (MNV-1) was discovered in 2003 as an enteric virus which affects immunocompromised mice and is within the same Norovirus genus as HNoV (Karst et al., 2003). MNV-1 shares many pathophysiological symptoms, biochemical as well as genetic features with HNoV. MNV-1 measures 28 to 35 nm in diameter, non-enveloped and icosahedral in shape, with a single-stranded, 7.5 kb RNA and three ORFs, representative of the two genera Noroviruses and Vesiviruses, that are within the *Caliciviridae* family (Wobus et al., 2006). MNV-1 binds glycolipids and glycoproteins on murine macrophages (Taube et al., 2012). MNV-1 displays unusual tropism for macrophages and dendritic cells and thus can be successfully replicated and propagated *in vitro* using RAW 264.7 macrophage cells (Wobus et al., 2004). MNV-1 is resistant to chemical treatments, where 2% TSP for 1 min at room temperature reduced MNV-1 (at initial ~7 log PFU/ml) by 1.05 log PFU/ml, unlike FCV and MS2, which

were reduced by more than 6 log PFU/ml (D'Souza and Su, 2010).Thermal treatments of MNV-1 are described in Table 1.5. MNV-1 showed 0.2and 2 log PFU/ml reduction in infectivity using 100mM citric acid (pH 2) and 100mMcarbonate buffer (pH 10) for 30 min at 37 °C, while there was 1.4 log PFU/ml reduction (from ~7 log PFU/ml) with commercial bleach at 1,000 ppm chlorine min at room temperature after 5 min treatment on inoculated stainless steel discs (Cannon et al., 2004; Cromeans et al., 2014). These studies suggest that MNV-1 might be a suitable surrogate for HNoV. However, it is highly sensitive to UV and shows complete reduction (from ~7 log PFU/ml) on surface of blueberries at 254 nm for 2 min (Liu et al., 2015).

3.3 Tulane Virus (TV):

Tulane virus was recently isolated from rhesus macaques (*Macaca mulatta*) stools (Farkas et al., 2008). TV belongs to the genus Recovirus in the *Caliciviridae* family, and is nonenveloped, icosahedral 40 nM small, with a positive sense, single stranded 7.5kb RNA genome (Farkas et al., 2008). HNoV and TV, both have three ORFs wherein ORF1 encodes non-structural protein, ORF2 encodes capsid protein (VP1) and minor structural proteins (VP2) are encoded by ORF3 (Farkas, 2015). The capsid protein containing 90 dimers are divided into the Shell domain (S) and the protruding domain (P), divided into subdomains namely P1 and P2, that are responsible for the entry of TV into the host cell (Farkas, 2015). Similar to HNoV, TV binds to histo-blood group antigens and therefore can be considered to be related more closely to HNoV GII (Farkas, 2015) making it a suitable surrogate for HNoV. TV can be cultivated *in vitro* using confluent Rhesus Monkey Kidney Epithelial Cells (LLC-MK2) as reported to be used in several studies (Farkas et al., 2008; Hirneisen and Kniel, 2013; Ailavaidi et al., 2019). D-values of TV in buffered cell culture medium in 2 ml glass vials at 52 to 58^{o}C were 4.59±0.05 to 0.58±0.02 min,

respectively using first order linear model (Ailavadi et al., 2019), lower than HAV which were 56.22±1.95 to 0.88±0.11 from 50 to 72^{o}C (Bozkurt et al., 2014). Treatments of TV- inoculated blueberries with gaseous ClO_2 generated using0.1 mg sodium chlorite decreased TV (~7 log PFU/ml) by >1 log PFU/ml after 30min and >2.2 log after 15 min treatment times (Kingsley et al., 2018). TV (at ~ 7 log PFU/ml) in cell-culture media showed 0.2 log PFU/ml reduction in infectivity using 100 mM citric acid (pH 2) and 100 mM carbonate buffer (pH 10) for 30 min at 37 °C, less than 0.5 log PFU/ml using 70% ethanol for 1 min and ~0.3 log PFU/ml when treated with 200 ppm chlorine on stainless steel coupons (Cromeans et al., 2014). Thus, TV may be suitable HNoV surrogate in some cases such as alcohol and chlorine treatments and not as suitable for use as a surrogate in other cases including high pressure processing.

3.4 RNA Coliphage (MS2) and Virus Like Particles (VLPs):

Bacteriophage MS2 contains a single-stranded RNA and has an icosahedral capsid, which is 27 to 34 nm (Strauss and Shinsheimer 1963), of group I in the *Leviviridae* family and commonly found in sewage water. As *E. coli* is the natural host of MS2, it is well adapted to intestinal tract and therefore used as a surrogate for HNoV and enteric viruses to study survival, disinfection, and inactivation (Dawson et al., 2005). 5 % TSP showed ≥6 log PFU/ml reduction for MS2 after treatment times of merely 30 sec or 1 min (D'Souza and Su, 2010). D-value of MS2 was 3 min at 37 ^{o}C on leafy salad vegetables (Allwood et al., 2004). Free chlorine at 10 ppm caused up to 2 log reduction of MS2 (~4 log PFU/ml) on strawberries after 10 min (Casteel et al., 2008).

Virus-like particles (VLPs) have similar structural and functional properties of their virus, however, they lack viral RNA making them non-infectious, and considered by some as better surrogates than bacteriophages or animal viruses that are typical surrogates used for inactivation

studies (Loisy et al., 2005; Hirneisen et al., 2010). Norovirus VLPs have been successfully produced as surrogates, however, they lack stability in survival and inactivation studies (Ausar et al., 2006). HNoV VLPs (38 nm) were completely disrupted at 700, 800, and 900 MPa aftertreatment times of 45, 15, and 2 min, respectively suggesting that HNoV capsid is highly resistant to pressure (Lou et al., 2012). At acidic conditions (pH 3), disruption in secondary structures of GI.1 VLP was 40% whereas GII.4 remained unaffected at high temperature of 75 ^{o}C, secondary structures of VLPs (GI.1 and GII.4) were disrupted by ~40 %, as analyzed by circular dichroism spectroscopy. This shows that acidic conditions (gastric conditions) may not cause any disruptions in HNoV capsid but heating at high temperatures can inactivate the capsid (Samandoulgou et al., 2015).

4. Surrogates for HAV:

The Australian strain of HAV, HM175 has been used to study biological properties, replicative strategies of virus, establish assays for detection and quantification as HM175 can be successfully propagated *in vitro* using Fetal Rhesus Monkey Kidney (FRhK-4) cell monolayers (Gust et al., 1985). However, recovery and enumeration of this strain using cell culture techniques takes several days and pose a challenge in industrial settings. Therefore, bacterial surrogates for HAV are investigated.

Listeria innocua is a Gram-positive, non-pathogenic rod-shaped bacteria found everywhere in the environment, in soil and food, and used as surrogate for *L. monocytogenes* in pasteurization (Busta et al., 2003; Friedly et al., 2008). In buffer, *Listeria innocua* at 65 °C had low D-values of 1 to 1.5 min at 58 °C compared to D-value of HAV (Renix, 2015; Friedly et al., 2008), and was not a suitable surrogate for HAV. *Lactobacillus bulgaricus* ATCC 11842 showed

slightly higher D-values that ranged from “9.98 min to 0.45 min at 65 to 70 °C” as reported earlier, but is an anaerobic or microaerophilic microorganism, that is not easily cultivated and enumerated (Renix, 2015). It requires conditions that may not be suitable for routine use for validation studies.

Another potential HAV surrogate is *Staphylococcus carnosus*, that is a Gram-positive, non-spore forming, non-pathogenic, non-motile, aerobic bacteria isolated from dry sausage and used as starter culture in sausage fermentation and termed as ‘food-grade’ with a broad temperature growth range from 15 to 45 ^{o}C (Götz et al., 2006; Schmidt, 2016). A previous study with *Staphylococcus carnosus* strain CS 299 grown at 32 ^{o}C reported D-values in phosphate buffer saline (PBS) within 2 ml glass vials of 1.59±0.20 to 0.36±0.07 min from 65 to 70 ^{o}C which increased to 3.13±0.48 to 0.5±0.03 min from 65 to 70 ^{o}C when grown at 40 ^{o}C (Schmidt, 2016). This study also reported that CS 299 grown at 32 ^{o}C gave D-values in milk of 2.57±0.08 to 0.43±0.08 min, 3.13±0.59 to 0.46±0.14 min in spinach, 2.83±0.48 to 0.46±0.04 min in mussels and 3.02±0.09 to 0.49±0.04 min in clams from 65 to 70 ^{o}C (Schmidt, 2016). Similar to D-values in PBS, when CS 299 was grown at higher temperature of 40 ^{o}C, the D-values at 70 ^{o}C was increased mostly in spinach and clams, with D-values of 0.44±0.09 min in milk, 1.24±0.04 min in spinach, 0.31±0.06 min in mussels and 0.82±0.02 min in clams using the linear model (Schmidt, 2016).

According to this data, *S. carnosus* CS 299 has much lower resistance to thermal treatments than that of HAV in buffer. However, $D_{65}{}^{o}{}_{C}$ of CS 299 grown at 40 ^{o}C in spinach was 1.24±0.04 min comparable to $D_{65}{}^{o}{}_{C}$ of HAV in buffer of 1.73±0.98 min, though it was higher in spinach of 2.30±0.82 min, concluding that *S. carnosus* when used in spinach may be a potential

surrogate for HAV (Schmidt et al., 2016; Bozkurt et al., 2014). Another strain of *S. carnosus* CS 300, may have higher heat resistance than CS 299 as it is a mixture of two strains of *S. carnosus* and reportedly has higher genetic stability than *S. aureus* (Löfblom et al., 2017). Hence, it is important to determine the heat-inactivation kinetics of this strain for potential use in validation studies for the inactivation of HAV.

5. Thermal inactivation:

Heat is traditionally used to inactivate most foodborne pathogens and enzymes present in foods at a given temperature for an appropriate time in order to ensure food safety and stability (Bozkurt et al., 2014). Selection of time-temperature regimes for thermal inactivation depends on magnitude of microbial inactivation, maintaining sensory characteristics, nutritional profile and shelf-life of product (Cebrian et al., 2017). For example, pasteurization treatments aim to inactivate foodborne pathogens up to 6 logs in order to extend shelf life of the food product under refrigeration conditions (Cebrian et al., 2017) and sterilization treatments are applied in order to inactivate foodborne pathogens (*C. botulinum*) by 12 logs to ensure food product stability at room temperature (Singh et al., 2018). In addition, sterilization of food requires higher temperatures which can inactivate spores of foodborne pathogens, spoilage and toxic enzymes. However, caution must be taken/used to ensure that the nutritional and sensory profile of food products are not lost due to such high time-temperature regimes (Cebrian et al., 2017).

Degree of heat resistance of microorganisms differ widely due to difference in their structure and composition and mechanisms of resistance. In order to evaluate heat resistance of different microorganisms at a certain temperature, parameter D-value or D_T is used which is defined as time required to obtain a one log reduction or 90% of a target population at a given

temperature (Cebrian et al., 2017). In addition to the D-value, z-value measures the difference in the temperature required in order to cause a 1 log difference in the D-value (Bozkurt et al., 2014). These values are useful for testing the effectiveness of thermal inactivation processes under varied conditions and situations, for example during cooking and preservation of foods (Gould et al.,1989).

Bigelow and Esty showed that the D-value is a negative inverse of slope obtained when logarithmic values of microbial survivors were plotted against treatment time. The equation thus obtained is based thermodynamic principles similar to the Arrhenius equation (Bozkurt et al., 2015). However, this model does not fully report heat inactivation. Hence, there are deviations present in linear survival curves like shoulder and tail phenomenon, that are extremely crucial in order to evaluate safety of foods (Mackey et al., 1987). "The shoulder effect can be associated with delayed response of microorganism to thermal treatments due to injury, while the tailing effect can be attributed to shielding of microorganisms by external particles" present in the medium, clumping of microorganisms and resistance **(**Mackey et al., 1987). These deviations are important in identifying and studying heat resistance of microorganisms and to determine changes in thermal treatments based on shoulder and tail effects.

Due to the above inconsistencies in the linear model for thermal inactivation, various other primary and secondary models have been studied (Cebrian et al., 2017). Unlike linear model, the Weibull model takes into account cumulative resistance of microbial survivors for a certain time period at a specific inactivation temperature (van Boekel et al., 2002). It is given by the equation-

$$\log \frac{N(t)}{No} = -b \times t^{\beta}$$

in which parameter b is defined as-

$$b = \frac{1}{2.303} \times (1/\alpha)^{\beta}$$

where α and β are the scale and shape parameters, respectively (Bozkurt et al., 2015). Where, β= 1corresponds to a linear relationship between microbial inactivation with respective to time. A value for β< 1 implies that the cells become sensitive to applied thermal treatment whereas β>1 suggests that the remainder cells become resistant to applied thermal treatment (van Boekel, 2002; Bozkurt et al., 2015).Geeraerd model is another thermal inactivation models which provides good fit to a variety of curves obtained during microbial death (Geeraerd et al., 2005). It is given by formula-

$$\frac{d\,N\,(t)}{dt} = -k_{max}\,(T\,(t))\,\frac{1}{1+Cc\,(t)}\,(N\,(t) - N_{res})$$

in which k_{max} is maximum inactivation rate, (min^{-1}), "N(t) (CFU/ml) represents microbial cell density at time t, N_{res} (CFU/ml) denotes the residual population density and Cc describes the physiological state of the cells" (Geeraerd et al., 2005; Zhu and Chen, 2016). In addition to primary models, secondary models are used to describe the change in inactivation parameters with changing environmental conditions including temperature of treatment, medium water activity and the treatment medium pH (Peleg et al, 2006).

5.1 Factors affecting heat inactivation:

Factors that affect treatment processes include, growth phase, growth temperature, growth media and exposure to sublethal stresses (Cebrian et al., 2008). *Growth phase*: Cells in the growth phase typically have higher sensitivity to thermal treatments than cells in stationary phase (Pagan et al., 1999). This is because alternative sigma factors which control genes involved in stress resistance are increased upon entrance into stationary phase(Pagan et al, 1999). *Growth*

temperature: Thermophiles have higher heat-resistance than mesophiles, and psychrophiles because their cellular proteins denature at higher temperatures (Sawle and Ghosh, 2011).Modification of the composition of fatty acid and protein of microbial cell membrane amounts to their tolerance to heat (Cebrian et al., 2017). Moreover, higher temperature of growth shows that cells have higher heat shock protein (HSPs) content, and their correct folding as well as elimination and repair of cell proteins that could be potentially damaged by heat, can also aid in thermostabilizing of membrane (Jay, 1992).*Growth medium*: Complex growth media also can increase microbial heat resistance (Cotterill, 1969). If the medium has lower water activity or lower pH, they protect cells against heat treatment. This is because, they stress adaptation responses are triggered in bacteria thereby making them heat resistant. A study conducted by Tolkier-Nielson et al, 1996 concluded that magnesium presence in treatment medium protects ribosomes and increased the heat resistance of *S.* Typhimurium. Thus, loss of magnesium due to membrane damage during heat treatment may cause destabilization of ribosomes and ultimately, cell death. *Exposure to sublethal stresses*: Defense mechanisms against various stressors including genetic regulation of heat shock response can be developed by microbial cells (Nonaka et al., 2006). *Water activity of treatment medium*: Water activity is universally defined and calculated by using "partial vapor pressure of water in a substance/commodity divided by the standard state partial vapor pressure of water analyzed at the same temperature" (Goepfet et al., 1987).Decrease in water activity (a_w) increases D- value in microorganisms, as stability of proteins increases in low a_w environment leading to increase in thermal resistance of microbial cells. Cells suspended in a high-osmolarity media show rapid intracellular water loss while and low water activity increases heat stability of proteins (Goepfert et al., 1987). *E. coli* O157:H7

and *S.* Enteritidis were reported to show increase in D-values from 0.5 min to 2 min and 2.5 min to 8 min, respectively at 59.5 ^{o}C using 0.5% w/w to 8.5% w/w NaCl (Blackburn et al., 1997). *pH of the treatment medium*: Acidification has been used to obtain safe and stable food products. When heated in acidic media, vegetative cells undergo rapid cytoplasmic acidification due to permeation of cell membrane, which results in rapid denaturation of proteins inside cells (Cebrian et al., 2017). Thus, microbes are more heat-resistant at near the neutral pH range sensitive to heat at acidic pH (Hassani et al., 2006**).** Acid-adaptation of *E. coli* in TSB at pH 5 significantly increased $D_{60^{o}C}$ in orange juice (OJ) from 1.1±0.35 to 1.7±0.34 min. Similarly, $D_{60^{o}C}$ of *L. monocytogenes* in orange juice (OJ) where acid adaptation of pH 5 increased 0.21±0.02 to 0.38±0.09 min (Mazzotta et al., 2001). *Composition of treatment medium*- Salts in the complex medium typically increases bacterial heat resistance (Stringer et al., 2000). Salts containing divalent cations (magnesium and calcium) reportedly stabilize outer membranes, molecular chaperones, ribosomes, and DNA (Stringer et al., 2000). $D_{63^{o}C}$ of *Salmonella* senftenberg in whole milk increased to 1.2 min as opposed to 0.37 min in phosphate-citrate buffer due to presence of divalent cations in whole milk (Manas et al., 2008). *External factors:* These are heavily influenced by environmental conditions and give rise to sublethal heat injury, that include lower temperatures during recovery, composition of recovery media such as divalent cations which exert protective effect to microbes, lower pH of the recovery media and atmosphere during recovery. Heated *E. coli* O157:H7, *Salmonella* Enteritidis and *L. monocytogenes* cells were reported to show improved recovery under low redox-potential and anaerobic conditions, in the presence of glutathione which is a reactive oxygen species (ROS) quencher (Cebrian et al., 2017). Heat resistance of *E. coli* (~7 log CFU/ml) increased with

glutathione (10 mM), where 0.95 log CFU/ml was inactivated at 58 ºC after 3 min as opposed to 2.83 log CFU/ml in PBS (Marcen et al., 2017). *Effect of heat treatment on cellular structures*- The bacterial cell membrane structure and composition as well as nucleic acid (DNA) content play a role in determining heat resistance. In Gram-negative bacteria, heat damages the outer lipopolysaccharide membrane of cells that causes membrane integrity loss and increase in sensitivity to antimicrobial compounds like bile salts, lysozyme or hydrophobic antibiotics (Mackey et al., 2000; Tomlins et al., 1976; Lee and Kaletunc, 2002). Due to increase in temperature around cell environment during heat treatment, microbial cell membrane is disrupted which leads to changes in internal homeostasis of the cell, alterations in cellular pumps, respiration activity, and loss of homeostasis (Teixeira et al., 1997).

DNA is one cellular component with high thermostability among cellular components, and can be denatured at sterilization temperatures, with dry heat (Cebrian et al., 2017). However, heat exposure makes DNA more susceptible to endonucleases causing subsequent damage and strand-breakage denaturation. Mackey et al, 1991 showed that *recA*, associated with DNA maintenance, *recB* which initiates recombinational repair from lethal breaks in double stranded DNA and *polA*, associated with DNA replication, indicating that heat injures DNA, by disrupting DNA repair enzymes (Cebrian et al., 2017).

Proteins are present in bacteria either as functional enzymes or structural proteins. Heat induces protein denaturation causing heat-treated cells to appear granular in structure. Protein denaturation can lead to loss of functions by inhibiting transport pumps, channels, DNA repair enzymes, that play important roles in cell recovery (Nguyen et al., 2006).

RNA and ribosomes are believed to be more sensitive than DNA to thermal treatments and thus believed to be critical targets involved in microbial inactivation (Earnshaw et al., 1995). Thermograms were obtained from whole *E. coli* and *L. plantarum* by Differential Scanning Calorimetry (DSC) from 1 to 150 ^{o}C at heating rate of 3 ^{o}C/min to compare untreated (control) and heat-treated cells at varying intensities to identify components that were affected (Lee and Kaletunc, 2002). This study suggested that irreversible denaturation of the ribosomes and subunits α and β of the RNA polymerase occurs at inactivation temperature (Nguyen et al., 2006). In addition to heat treatments direct effects on components of the cell, accumulation of reactive oxygen species (ROS that include single oxygen species, hydroxyl radical, superoxide and peroxide) can be detrimental to microbial cells. ROS is generated due to destabilization of cellular structures, or decreased bacterial defense system activity when microbial cells are subjected to heat treatments (Earnshaw et al., 1995). These molecules react with cellular components like DNA, proteins and also lipids from cellular membrane (Marcein et al., 2017).

6. Non-thermal inactivation:

In addition to thermal inactivation, novel non-thermal approaches to enhance food safety and maintain nutrient value and sensory attributes are also being researched. These include High hydrostatic pressure (HHP), Electron beam, photodynamic inactivation (PDI), Chlorine dioxide (ClO_2 gas) treatment, natural antimicrobials and others.

6.1. High hydrostatic pressure (HHP):

High Pressure processing (HHP) permits microbial inactivation at moderate to low temperatures, less than 50 ^{o}C thus preserving nutrient and sensory profile of the foods. Derived from the field of material sciences, food matrices are subjected to high pressure of 100-1000 MPa that is, 1000

to 10,000 bars which reduces contamination from foodborne pathogens while maintaining sensory and nutritional attributes (Cheftel, 1995; Li et al., 2013). Also, such high pressure may result in loss of receptor-binding ability of viruses (Lou et al., 2011). One study conducted on HNoV GI.1 and GII.4 HNoV inoculated into oyster homogenates showed >4 log reduction in RNA level of both GI.1 and GII.4 at 600 MPa at 6 °C, and 0.7 and 1.3 log reduction in RNA level of GI.1 at 300 and 400 MPa, respectively at 6 °C and 2.9 and 3.6 log reduction in RNA level of GII.4 at 300 and 400 MPa, respectively at 6 °C (Ye et al., 2014). FCV-F9 was decreased by 5 and 4 log PFU/ml at 200 MPa at -10 and 50 °C, respectively after 4 min, and decreased by a 4 log PFU/ml after treatment for 7 min at 59.3 °C (Kingsley et al., 2007). HAV titer levels were decreased by >1, >2, and >3 log PFU/ml after 1 min treatment at 350, 375, and 400 MPa HHP, respectively, at temperatures of 8.7 to 10.3 °C (Calci et al., in 2005). In culture medium, HAV (~ 7 log PFU/ml) was non-detectable at > 450 MPa for 5 min (Kingsley et al., 2002).

6.2 Electron beam:

This technique uses high-speed electrons, accelerated by a machine for production of irradiation which disrupts viral structure and degrades viral RNA and proteins (Zhou et al., 2011; Predmore et al., 2015). Irradiating fresh-cut lettuce and spinach up to "4 kGy as a pathogen kill step has been approved by US FDA" (Espinosa et al., 2012). It was observed that the E-beam dose decreasedMNV-1 and HAV (from initial titers of ~7 log PFU/ml) by 90% in whole oysters (*Crassostrea virginica*) was 4.05±0.63 and 4.83±0.08 kGy, respectively (Espinosa et al., 2012). One study claimed that 5-kGy treatment achieved a 12% reduction in HNoV (~ 5 log PFU/ml) and 16% reduction in HAV (~ 5 log PFU/ml) respectively at high titers and 26% reduction of NoV (~2 log PFU/ml) and 91% reduction of HAV(~2 log PFU/ml) respectively at low titers in

raw oysters (Praveen et al, 2013). In case of TV (~ 6.6 log PFU/ml) in cell culture media, 8.6 and 16.9 kGy e-beam reduced TV to 1.8 log PFU/ml and 3.2 log PFU/ml, respectively (Predmore et al., 2015). In the case of lettuce and strawberries inoculated with TV at 3.7 and 4.4 log PFU/ml, respectively, 8.7 kGy e-beam was reported to reduce TV to non-detectable level for lettuce and 1.8 log PFU/ml for strawberries. This shows that e-beam was more effective in reducing the virus in food matrix as compared to cell culture media, but the dosage required for reduction of TV to non-detectable level was higher (8.7 kGy for lettuce and 16.9 kGy for strawberries) than permissible limits approved by FDA (Predmore et al., 2015).

6.3. Chlorine dioxide (ClO_2):

Chlorine dioxide (ClO_2) is an antimicrobial gaseous oxide of chlorine used to disinfect water, sanitize equipment and surfaces in food industry and bleach flour and paper (Keskinen and Annous, 2011). It has been shown that ClO_2 gas can inactivate bacterial pathogens and spores at optimized concentration, relative humidity, and temperature. Due to its high efficacy against bacterial spores, ClO_2 gas has been approved by the EPA as a decontaminant for emergency anthrax (*Bacillus anthracis* spores) (EPA, 2012). Also, yeasts and molds associated with food safety and spoilage are reported to be inactivated by ClO_2 gas (Trinetta et al., 2011). ClO_2 gas treatment of 10 mg/L, 3 min, 75% RH is able to effectively inactivate *Salmonella enterica* and *E. coli* O157:H7 on tomato, cantaloupe, and lettuce seeds with minimal impairment to their subsequent germination (Trinetta et al., 2011). For fresh produce, ClO_2 gas ranging from 0.1 mg/L to 10 mg/L and exposure time of 3 min to 14 min were effective not only in reducing the burden of *S. enterica* and *L. monocytogenes* contamination on fresh produce, but also improved the shelf life (Bhagat et al., 2010, Trinetta et al., 2010). Antiviral properties of ClO_2 gas against

MNV-1 have been investigated on stainless steel coupons where ClO_2 gas at 2 mg/L resulted in minimal 3-log reduction in MNV-1 (at initial titers of ~7 log PFU/ml) after treatment time of 5 min and 2.5 mg/L after 2 min and no infectious virus was recovered when concentration was increased to 4 mg/L at 25 ^oC after 1 min. It was elucidated that ClO_2 gas inactivates virus by degradation of viral protein and viral genomic RNA and, viral structure disruption, (Yeap et al., 2016).

6.4 Photodynamic inactivation (PDI):

"The mode of action of PDI relies on the principle that photosensitizers (PS) can absorb energy from light of particular wavelengths and transfer that energy to molecular oxygen to form reactive oxygen species (ROS) which kills microbial cells by cytotoxic reactions" (DeRosa et al, 2002).This technique has been specifically used for decontamination of food contact surfaces and not food matrices. Photoactivated curcumin (5 µg/ mL) at 450 nm reduced FCV titer (~7 log $TCID_{50}$ /ml.) by 1.75 log and 4.43 log at RT and 37 ^oC, respectively and MNV-1 by 0.73 log at 37 ^oC respectively after 30 min (Randazzo et al., 2016). Curcumin at 10 µM and 20 µM, caused MNV-1 reductions of 0.76 log PFU/ml and 1.15 log PFU/ml reduction, respectively in oysters (Wu et al., 2015).

7. GRAS compounds in food processing:

Foodborne illness and food spoilage are challenges that have been well established in public health and the food industry. In 2011, the U.S. CDC (Centers for Disease Control and Prevention) estimated that the U.S. population experiences "48 million cases of foodborne illness annually, resulting in almost 128,000 hospitalizations and 3,000 deaths" (CDC, 2018) at a cost of billions of dollars every year within the food industry (Enderson et al., 2014). Food industry

leaders must find methods to tackle these issues while also appealing to consumer demands to use less chemicals and more naturally derived compounds in foods. One of the most well-known and popularly used antimicrobials is benzoic acid, obtained from berries, including those of the *Vaccinium* species like cranberry, blueberry, bilberry, lingonberry and huckleberry (Cagri et al., 2004; del Olmo et al., 2017). Benzoic acid is used in food processing mainly for its antimicrobial properties against yeasts/fungi, however it is also able to inhibit the growth of Gram positive and Gram negative pathogenic/spoilage bacteria (Cagri et al., 2004). Gram positive bacteria are quite susceptible to the effects of benzoic acid and its derivates like potassium and sodium benzoate. It is considered GRAS at levels up to 0.1% of food (Cagri et al., 2004; Damodaran et al., 2017). Another notable and well-established antimicrobial is sorbic acid which is produced commercially using ketene and crotonaldehyde (Lück et al., 1997). Sorbic acid, and one of its salt derivatives, sorbate, are perhaps the most commonly used and popular antimicrobials against fungi and bacteria like *S.* Typhimurium, *E. coli*, *Staphylococcus, Pseudomonas, Vibrio parahaemolyticus,* and *Bacillus* (Lück et al., 1997; Sofos et al., 1991). Lysozyme is a monomeric enzyme which has also been used for many years in food processing. It and its antimicrobial properties were discovered in 1922 by Alexander Flemming by testing his own nasal mucus. Lysozyme is present in many animal tissues and bodily secretions, but the source for commercial use is chicken eggs, known as HEWL (hen egg white lysozyme). It is used against *Listeria monocytogenes* in many commodities such as seafood, kimchi, Chinese noodles, potato salad and hard cheeses (Masschalck et al., 2003). Nisin, a bacteriocin polypeptide obtained from *Lactococcus lactis* spp. With GRAS food additive status in more than 50 countries (Lucera et al., 2012) It displays antimicrobial action against heat-resistant *C. botulinum* spores and against

food-borne pathogens such as *L. monocytogenes*, *Staphylococcus aureus,* and *Bacillus cereus* (Brewer et al., 2002; Lopez-Pedemonte et al., 2003; Sobrino-Lopez and Martin-Belloso, 2006). Allyl-isothiocyanate (AITC), is another plant extract used against *E. coli* (Nadarajah et al., 2002; Muthukumarasamy et al., 2003).). A microbial sachet that incorporated AITC showed antimicrobial activity against *Staphylococcus* sp. and psychrotrophic bacteria reducing *Staphylococcus* sp. by 2.4 log CFU/ml after 12 days whereas yeasts and molds showed 3.6 log CFU/ml reduction after 15 days in sliced mozzarella cheese stored at 12±2 °C (Pires et al. 2009).

Curcumin is a phytochemical derived from the rhizome of *Curcuma longa L.* belonging to the *Zingiberaceae* family. It is a diferuloylmethane (1,7-bis (4-hydroxy-3- methoxyphenyl)-1,6-heptadiene-3,5- dione), commonly known as turmeric (Monghadamtousi et al., 2014). Traditionally used as a food color in Asian food preparations, this polyphenolic compound has gained significant attention in recent years due to its variety of biological activities (Monghadamtousi et al., 2014). In addition to anti-inflammatory effects of curcumin, previous studies have investigated its antibacterial and antifungal properties against foodborne pathogens such as *S. aureus, V. parahaemolyticus, C. albicans* (Monghadamtousi et al., 2014) and against oral biofilms (Quishida et al., 2016). Besides these, antiviral properties of curcumin have been studied against *Picornaviridae* (Enterovirus 71(EV71)) (Qin et al.,2014), *Caliciviridae* (Yang et al., 2016) and against HNoV surrogates where curcumin at 5 µg/mL decreased FCV titers by 1.75 log $TCID_{50}$/ml at room temperature after 30 min (Randazzo et al., 2016). Curcumin at 2 mg/ml was reported to neutralized MNV-1 by 90.43% ±9.13%, at 4 ^{o}C after 3 days (Yang et al., 2016).

Grape seeds obtained from *Vitis vinifera* contains a mixture of antimicrobial and anti-inflammatory compounds like proanthocyanidins, catechin, epicatechin and epicatechin-3-O-gallateand gallic acid (Saada et al., 2000; Nassiri-Asl et al., 2016). Saada et al., (2000) demonstrated that 100 mg/kg of grape seed extract (GSE) when fed to rats, reduced gamma-radiation-induced oxidative stress and protected vital organs including the heart and pancreas from damage associated with oxidation (Saada et al., 2000). In addition to anti-inflammatory, anti-proliferative and anti-diabetic effects of GSE, procyanidin, an active compound found in *V. vinifera*, was reported to have anti-influenza A activity (Nassiri-Asl et al., 2016). Commercially available GSE is sold as dietary supplement and listed as "Everything Added to Food in the United States" (EAFUS)" and has the status of "Generally Recognized as Safe (GRAS)" by the US FDA (Perumalla et al., 2010). GSE has demonstrated antimicrobial activity *in vitro* against foodborne pathogens including *Bacillus cereus, Enterobacter sakazakii, E. coli* O157:H7, *Aeromonas hydrophila, L. monocytogenes, S.* Typhimurium, *Staphylococcus aureus* (Perumalla et al., 2010; Al-Habib et al., 2010). GSE (2 mg/ml) has been evaluated for its antiviral properties for maintaining food quality and safety. GSE reduced MNV-1 (~5 logs PFU/ml) on lettuce by 1 log PFU/ml and on peppers by 1.2 logs PFU/ml after 5 min contact at room temperature and (Su and D'Souza 2013). GSE at 2mg/ml also reduced FCV-F9 (~ 7 log PFU/ml) by 4.61 log and MNV (~ 7 log PFU/ml) by 1.73 logs at room temperature after treatment for 2h (Su and D'Souza, 2011). Joshi et al., (2015) reported that 1 mg/ml GSE decreased MNV-1 from initial titers of ~ 5 log PFU/ml to non-detectable levels after 1 h in apple juice and by 1 log in 2% milk after 24 h. They also reported that 1 and 2 mg/ml GSE decreased HAV titers to nondetectable

levels after 1 h in apple juice, and 2 mg/ml GSE in 2% milk reduced HAV by 1 log after 24 h (Joshi et al., 2015).

Gingerol is a phenolic compound obtained from ginger or *Zingiber officinale.* It is a dried rhizome, having pungent flavor and hence used as a spice in the Indian subcontinent and in Southeast Asia. It is also known to treat headaches, cold and sore-throat (Cho et al., 2015). Gingerol has been studied for its pharmacological properties such as anti-nausea, anti-inflammatory, and anti-carcinogenicity (Aboubakr et al., 2016). Additionally, Gingerol at 12.5 µg/ml after 72 h has been reported to have antimicrobial effects causing 6 log CFU/ml reduction of *Helicobacter pylori*, while 20 µg/ml of gingerol caused a 5 log CFU/ml reduction of periodontal bacteria after incubation for 24 h (Mahady et al., 2003; Park et al, 2008). Gingerol at 20 µg/mL at 4 ºC for 3 days was also shown to neutralize MNV-1 by 43.77% ± 4.50% and inhibited replication of HNoV in replicon-bearing HG23 cells by 40.50% ± 3.83% (Yang et al., 2016).

As plant extracts have demonstrated their potential for increasing food safety, our hypothesis is that these extracts can aid in the thermal inactivation of viruses by decreasing the time-temperature requirement for their inactivation. Therefore, one objective was to compare the effects of plant extracts (GSE, curcumin and gingerol) on the thermal inactivation parameters of HAV and TV in buffer in 2-ml glass vials. This would provide data for potentially decreasing the temperature-time parameters for food processing to reduce energy and subsequent processing costs. Another hypothesis is that the heat-resistance of *S. carnosus* can be increased by modifying growth conditions. Therefore, the second objective was to determine the heat inactivation (D-values) of *S. carnosus* at higher temperature, low pH and increased salt. Thus,

the overall goals of this project were to (1) determine the temperature-time requirements for the inactivation of HAV in the presence of plant extracts present in foods; and (2) to determine appropriate bacterial surrogates for HAV that can be potentially for applied in thermal inactivation validation studies in foods.

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Appendix:

Table 1.1: Recent outbreaks of HAV in the US

Year	Sate	Contamination source	Reference
2003	Pennsylvania	Green onions	Wheeler et al., 2005
2013	Multistate	Pomegranate seeds	Collier et al., 2014
2016	Hawaii	Raw scallops	Viray et al., 2018
2016	Multistate	Frozen strawberries	Chatziprodromidou et al., 2018
2018	Multistate	Improper hygiene, sharing needles	O'Neil, 2018

Table 1.2: Recent outbreaks of HNoV in the US

Year	Sate	Contamination source	Reference
2011	Multistate	Cantaloupe	Matthews et al., 2012
2011	Oregon	Strawberries	Chatziprodromidou et al., 2018
2012	Multistate	Cantaloupe	Walsh et al., 2014
2012	Multistate	Ricotta salata cheese	CDC, 2018
2012	California	Soup mushroom	Robilotti et al, 2015
2014	Multistate	Caramel apple	CDC, 2018
2014	Multistate	Mung bean sprouts	CDC, 2018
2015	Ohio	Homemade potato salad	CDC, 2018
2015	North Carolina	Pork	CDC, 2018
2015	Florida	Raw oysters	Woods et al., 2016
2015	Multistate	Prepackaged lettuce and cucumber	Grove et al., 2016
2015	Massachusetts	Chipotle restaurant	CDC, 2018
2018	Texas	Cruise ship	Cannon et al., 2017

Table 1.3: Thermal inactivation kinetics of HAV

T (ºC)	Medium	Experimental Vessel	Time	D-value (min)	Log Reduction	Unit	Reference
50	Cell culture medium	2 ml glass vials		56.22±1.95			Bozkurt et al., 2014
56				8.40±0.4			
60				2.67±0.42			
65				1.73±0.98			
72				0.88±0.11			
50	Mussels	2 ml glass vials		54.17±4.94			Bozkurt et al., 2014
56				9.32±3.26			
60				3.25±0.72			
65				2.16±0.17			
72				1.07±0.24			
85	Soft Shell Clams	Stomacher bags	90 s		1.33	PFU/ml	Sow et al., 2011
90	(*Mya arenaria*)		180s		4.75	PFU/mL	
			300s		5	PFU/mL	
50	Spinach	Vacuum bags		34.40±4.08			Bozkurt et al., 2015
56				8.43±1.72			
60				4.55±0.82			
65				2.30±0.82			
72				0.91±012			

Table 1.3: Thermal inactivation kinetics of HAV (continued)

T (ºC)	Medium	Experimental Vessel	D-value (min)	Reference
50	Turkey deli meat	Vacuum bags	42±5.6	Bozkurt et al., 2015
56			20.6±2.5	
60			5.9±1.3	
65			2.3±0.4	
72			1±0.1	

Table 1.4: Thermal inactivation kinetics of MNV

T (ºC)	Medium	Experimental vessel	D-value (min)	Reference
50	Cell culture medium	Capillary Tube	34.49±2.10	Bozkurt et al., 2013
56			3.65±0.005	
60			0.57 ± 0.01	
65			0.30 ± 0.00	
72			0.15 ± 0.00	
50	Cell culture medium	2 ml glass vials	36.28±3.21	Bozkurt et al., 2014
56			3.74±0.68	
60			1.09±0.03	
65			0.77±0.03	
72			0.25±0.01	
50	Spinach	2 ml glass vials	14.57±2.89	Bozkurt et al., 2014
56			3.29±0.96	
60			0.98 ± 0.24	
65			0.40±0.22	
72			0.16±0.11	

T (ºC)	Medium	Experimental vessel	D-value (min)	Reference
50	Blue Mussels (*Mytilus edulis*)	2 ml glass vials	20.19±0.22	Bozkurt et al., 2014
56			6.21±0.81	
60			2.64±0.15	
65			0.41±0.03	
72			0.18±0.03	
50	Turkey deli meat	Vacuum bags	21.0±0.8	Bozkurt et al., 2015
56			7.3±0.8	
60			2.7±0.6	
65			0.9±0.1	
72			0.2±0.0	

Table 1.4: Thermal inactivation kinetics of MNV (continued)

Table 1.5: Thermal inactivation kinetics of TV

T (ºC)	Medium	Experimental vessel	Time (min)	D-value (min)	Log Reduction	Unit	Reference
50	Cell culture medium	0.2 ml PCR tubes	2		1.79	PFU/mL	Hirneisen and Kniel, 2013
55					1.83		
60					2.9		
65					3.07		
56	Cell culture medium	Microcentrifuge Tube	20		3.5	$TCID_{50}$/mL	Cromeans et al., 2014
60			10		4		
56	Cell culture medium	Microcentrifuge tube		11.8			Cromeans et al., 2014
63				2.6			
72				4.3			
37	Cell culture medium	Microcentrifuge tubes		500			Arthur et al., 2015
56				4.03			
63				1.18			
72				0.24			
50	Cell culture medium	2 ml glass vials		4.59±0.05			Ailavadi et al., 2019
54				2.91±0.05			
58				1.74±0.07			
60				0.58±0.02			
50	Spinach	Vacuum bags		7.94±0.21			Ailavadi et al., 2019
54				4.09±0.04			
58				1.43±0.02			

CHAPTER II

HEAT SENSITIZATION OF HEPATITIS A VIRUS AND TULANE VIRUS USING PLANT EXTRACTS

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Running title: Decreased thermal resistance of hepatitis A virus and Tulane virus using plant extracts

Key words: Hepatitis A virus, Tulane virus, heat inactivation, first order linear model, Grape seed extract, curcumin, gingerol. (*For submission to Food Microbiology Journal*).

Abstract:

Human noroviruses (HNoV) and hepatitis A virus (HAV) are increasingly associated with human foodborne illness outbreaks throughout the world. As cell-culture systems to propagate HNoV in laboratory settings are not easily available, Tulane virus (TV) is used as a cultivable HNoV surrogate to determine inactivation. Heat-sensitization of HAV and TV by "generally recognized as safe'' (GRAS) substances can potentially reduce their time-temperature inactivation parameters during processing to ensure food safety. Curcumin, gingerol (from ginger), and grape seed extract (GSE) have reported anti-inflammatory, immune-modulating and antiviral properties. The objective of this study was to determine and compare the D-values of HAV and TV at 52 to 68 ^{o}C with or without curcumin (0.015 mg/ml), gingerol (0.1 mg/ml), or GSE (1 mg/ml). HAV at ~7 log PFU/ml and TV at ~6 log PFU/ml were diluted in phosphate buffer saline (PBS) and added to two sets of six 2mL sterile glass vials. One set served as the control, while the three extracts were individually added to the second set for thermal treatments in a circulating water bath for 0 to 10 min. The D-values for TV in PBS ranged from 4.59±0.28 to 1.08±0.16 min, and for HAV in PBS ranged from to 9.21±0.24 to 0.67±0.19 min at 52 to 68 ^{o}C. Decreased D-values of TV with curcumin ranged from 4.32±0.25 to 0.62±0.17 min, gingerol from 4.09±0.18 to 0.72±0.09 min and GSE from 3.82±0.18 to 0.80±0.07 min, with similar trends for HAV. The linear model showed significant differences ($P<0.05$) between the D-values of HAV and TV with and without plant extracts for most tested temperatures. GRAS substances can potentially lower temperature and time regimens needed to inactivate HAV and TV.

1. Introduction:

Currently, the majority of foodborne viral outbreaks in the world are linked to human noroviruses (HNoVs) which cause acute gastroenteritis in populations of all ages (Scallan et al., 2015; Hall et al., 2013). Hepatitis A virus (HAV) is another important foodborne enteric virus which causes severe liver disease, with symptoms that include jaundice (Scallan et al., 2015; Lemon et al., 2018). HNoV and HAV are spread through the fecal-oral route. Therefore, contamination of food can occur at any point or time from the farm to fork, during cultivation, harvesting, processing, distribution and storage (Acheson and Fiore, 2004). Due to the lack of good hygienic practices, the risk of HAV and HNoV illness spread has escalated in developing countries and also in developed countries due to import of sub-standard, food products that could be contaminated, and exposure of naive populations during travel (Koopmans and Duizer, 2004). Among the techniques used for foodborne virus inactivation, thermal processing is the most effective and widely used method to ensure food safety and to increase the shelf-life of a food product (Silva and Gibbs, 2012). Thermal inactivation studies on HAV in buffer revealed that HAV is more resistant than *Listeria monocytogenes* with a $D_{72}{}^{o}{}_{C}$ of 0.88 ± 0.11 min (Bozkurt et al., 2014). In the case of HNoV, their impact on public health and numerous associated outbreaks, makes them the most prevalent of all foodborne viruses. Recently, only certain genogroups/genotypes of HNoV were reported to be cultivated in the lab, though at low titers (Ettayebi et al., 2016). Therefore, cultivable viral surrogates including murine norovirus (MNV-1), feline calicivirus (FCV-F9), Tulane virus and porcine sapoviruses are used which mimic the inactivation characteristics of HNoV (Joshi et al., 2015; Su and D'Souza, 2011; Ailavadi et al., 2019; Esseili et al., 2018). Tulane virus (TV) belongs to the same *Caliciviridae* family as HNoV

and displays similar histo-group binding to HNoV, thus making it a suitable HNoV surrogate (Tian et al., 2013; Hirneisen et al., 2013). As these enteric viruses are highly persistent and resistant to traditional food processing techniques, better processing conditions are required in order to prevent and control their contamination and outbreaks. The food processing industry aims to achieve faster viral inactivation in lesser time to increase productivity and retain organoleptic attributes of food product. This can be achieved by decreasing the heat resistance of target pathogens by using hurdle technologies (WHO, 2013; Singh and Shalini, 2016). Combination of plant extracts with heat could be one such effort in this direction. Compounds that belong to the 'generally recognised as safe' (GRAS) category can be used as food additives to aid in food processing. They are secondary metabolites of plants and can sequentially inhibit several steps in a particular biochemical pathway, inhibit viral enzymes, interact with viral capsids, or lead to increased uptake of other antimicrobials (Zhou et al., 2007).

Some studies have investigated the effect of plant extracts on the heat resistance of foodborne pathogens (Cui et al., 2011; Bevilacqua et al., 2013; Juneja et al., 2008). Licorice (5% v/v) and pineapple sage extract (5% v/v) was shown to reduce *Clostridium botulinum* 62A spores by 6 logs when heated at 80 ^{o}C for 60 min and 5.56 log when heated at 100 ^{o}C for 60 min, respectively (Cui et al., 2011). Lemon extract (80 ppm) was reported to have heat sensitizing effects against spores of two strains of *Alicyclobacillus acidoterrestris* that showed reduction of 2 log CFU/ml after treatment at 80 ^{o}C for 6 min compared to 0.75 log CFU/ml reduction in the control (Bevilacqua et al., 2013). In foods including commercially available orange juice, the addition of 200 ppm (+)-limonene was shown to reduce the heating time by 3.8-fold at 54–60 °C to inactivate 5 log *Escherichia coli* O157:H7 (Espina et al.,2014). Furthermore, carvacol and

cinnamaldehyde were reported to have heat sensitizing effects against *E. coli* O157:H7 in raw ground beef, where trans-cinnamaldehyde (1% v/v) decreased the D-value of *E.coli* at 55 ^{o}C from 63.90±0.51 min to 11.81±4.41 min and carvacrol (1 % v/v) decreased the D- value at 55 ^{o}C from 63.90±0.51 min to 18.16±0.66 min (Juneja et al., 2008).

Grape seed extract (GSE), curcumin and gingerol have demonstrated antiviral activity (Su and D'Souza, 2011; Joshi et al., 2015; Yang et al., 2016; Aboubakr et al., 2016). Grape seed extract (GSE) is a wine industry by-product isolated from seeds of *Vitis vinifera* which is rich in proanthocyanins (Yang et al., 2016). GSE at 1 mg/ml was reported to have antiviral activity against hepatitis A virus, and also cultivable HNoV surrogates, feline calicivirus (FCV-F9) in viral suspensions causing decrease in titers by 3.20, 1.73 and 4.61 log PFU/ml respectively, and also decreased viral titers under simulated gastric conditions after 24 h at 37 ^{o}C and on produce (Su and D'Souza, 2011; Su and D'Souza, 2013; Joshi et al., 2015). Curcumin is the principal curcuminoid obtained from the rhizomes of turmeric or *Curcuma longa* that is extensively used as a spice in Asian food preparations (Aggarwal et al., 2007). In addition to its anti-inflammatory properties against cancer, curcumin displays antimicrobial properties against foodborne pathogens such as *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 10031 (Moghadamtousi et al., 2014). Curcumin (2 mg/ml) was shown to have effects against MNV-1 in suspension causing decrease in titers by 0.96 log PFU/ml after 7 days at 4 °C (Yang et al., 2016). Curcumin affected the envelope fluidity of hepatitis C virus (HCV) without changing the integrity of the virus that resulted in inhibition of binding and fusion to its host cells (Anggakusuma et al., 2014). Gingerol is a phenolic compound obtained from ginger or *Zingiber officinale* which has anti-nausea, anti-inflammatory, and anti-

carcinogenic properties, where 5% v/v gingerol was reported to decrease FCV titers in suspension tests by 2.7 log PFU/ml (Aboubakr et al., 2016) at room temperature or at 37 ^{o}C. Antiviral properties of GSE, curcumin and gingerol in the presence of heat are yet to be investigated. It can be hypothesized that these extracts may have synergistic or additive effects with heat making enteric viruses more sensitive to thermal inactivation, thereby reducing the actual time and temperature required for enteric viral inactivation. Therefore, the present study aimed to investigate the heat sensitizing potential of GSE, curcumin and gingerol against HAV and TV in phosphate buffer saline in 2-ml glass vials. The D- and z-values for these viruses with and without GSE, curcumin or gingerol were compared.

2. Materials and Methods:

2.1 Viruses and Cell lines:

Viruses and their respective host cells included hepatitis A virus (HAV; strain HM175) from Dr. Kalmia Kniel's laboratory (University of Delaware), fetal rhesus monkey kidney (FRhK-4) cells obtained from the UT collection, Tulane virus (TV) obtained from Dr. J. Jiang (Cincinnati Children's General Hospital, Cincinnati, Ohio) and host LLC-MK2 cells that were used for infection and propagation as described earlier (Su and D'Souza, 2013; Fino and Kniel, 2008; Ailavadi et al., 2019). FRhK-4 cells were maintained using standard published protocols at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle's medium/Ham's F-12 (DMEM-F12) that had 2% heat-inactivated bovine calf serum (BCS; HyClone Laboratories, Logan, UT) and also 1× antibiotic-antimycotic (ThermoFisher Scientific) added to the media as reported in earlier studies (Bozkurt et al., 2014). Previously described protocols were also used to maintain LLC-MK2 cells (Arthur and Gibson, 2015). Briefly, LLC-MK2 were maintained in Opti-MEM® I Reduced

Serum Medium (OptiMEM® GlutaMAX™ Supplement, Grand Island, NY) containing 1X Anti-anti and 2% heat inactivated BCS and incubated at 37 °C under 5% CO_2.

2.2 Propagation of viruses and quantification of virus titers:

FRhK-4 and LLC-MK2 cells that were approximately 90% confluent in cell culture flasks were first rinsed with Dulbecco's Phosphate Buffer Saline (DPBS, pH 7.4) and then HAV and TV stocks were added to their respective cell monolayers and incubated in a CO_2 incubator at 37 ^oC until >90% cell lysis for approximately 7 days for HAV or 3 days for TV (Joshi et al., 2015; Ailavadi et al., 2019). Both the viruses were obtained separately by centrifugation at 5000 x g for 10 min, and then 0.2 µm filters were used to filter the virus that was aliquoted and kept frozen at -80 ^oC until use. Stock of viruses were serially diluted and infected on confluent FRhK-4 cell monolayers (for HAV) and LLC-MK2 cell monolayers (for TV) in 6-well plates. The titers of HAV were approximately 7 log PFU/ml and TV titers were ~ 6 log PFU/ml.

2.3 Preparation of plant extracts:

GSE, Gravinol-S, was prepared as previously described (Su and D'Souza, 2011), that was giftedby OptiPure®, Chemco Industries (Los Angeles, CA). Briefly, GSE solutions were prepared by dissolving GSE in water and ethanol (5% v/v), that was passed through a 0.2 µm filter, and diluted aseptically to 10 mg/ml (pH 5.40) in phosphate buffered saline (DPBS, pH 7.4) (Su and D'Souza, 2011). Similarly, curcumin (Acros Organics, NJ) powder and gingerol (6-Gingerol; Cayman Chemical Company) were dissolved in ethanol (5% v/v) and water, passed through 0.2 µm filters and then aseptically diluted to 0.15 mg/ml curcumin and 1 mg/ml gingerol in phosphate buffered saline (DPBS, pH 7.4). All the extracts were stored at 4 ^oC until further use.

2.4 Thermal treatments:

Thermal treatments were carried out in a circulating water bath (ISOTEMP 2150, Fisher Scientific) in 2-ml screw-capped sterile glass vials as described earlier (Bozkurt et al., 2014; Ailavadi et al., 2019). One control set of sterilized glass vials was carefully aseptically filled with HAV or TV suspended in buffered cell culture medium (1:10). Another set of sterilized glass vials containing TV or HAV in buffered cell culture medium were aseptically mixed with plant extracts to obtain final concentrations of GSE (1 mg/ml), curcumin (0.015 mg/ml) or gingerol (0.1 mg/ml) in the tube. As described earlier, the surfaces of the filled vials were washed with 70% ethanol and then immersed in a waterbath that was thermostatically controlled (Bozkurt et al., 2014; Ailavadi et al., 2019). Briefly, thermocouple probes (MMS3000, Commtest Instruments) were immersed in such a way that one probe measured the temperature of the circulating water and the second probe measured the temperature at geometric centre of a sterile vial containing PBS to record the temperature inside the vial. Thermocouples were connected to a data recorder to measure temperatures. The 2-ml vials with HAV and plant extracts were subjected to 56, 60, 65 and 68 ^{o}C for 0 to 10 min. Whereas, the 2-ml vials with TV along with three plant extracts were subjected to 52, 54, 56, and 58 ^{o}C for a span of 0 to 10 min. In every experiment, after the come-up time was noted, then the treatment time began. After each of the thermal treatments that were carried out in triplicate, samples were cooled on ice immediately for 15 s to stop/prevent thermal inactivation as much as possible. These contents were then transferred to microcentrifuge tubes containing 1.5 ml DMEM supplemented with 8% BCS for HAV and 1.5 ml OptiMEM supplemented with 8% BCS for TV to be serially diluted before assaying as described below.

2.5 Enumeration of survivors:

Thermally treated HAV and TV were ten-fold serially diluted in 1.5 ml tubes containing DMEM and OptiMEM supplemented with 2% BCS, respectively, and standardized plaque assays were used to determine infectious viral titers. Suitable dilutions of heat treated HAV and TV were infected on 6-well plates of confluent FRhK-4 and LLC-MK2 monolayers, respectively following previously described protocols (Bozkurt et al., 2014; Ailavadi et al., 2019). Surviving viruses were enumerated by previously described plaque assays as plaque forming units (PFU/ml) where one plaque represented one virus on a 6-well plate (Bozkurt et al., 2014).

2.6 Statistical Analysis:

Recovered counts of plaques from each experiment carried out three times and assayed in duplicate were analysed. Plaque counts were converted into logarithmic values ($\log_{10}$ PFU/mL), to calculate D-values (MS Excel, Version 2017). As described earlier, significant differences of D-values between viral suspensions in PBS and in individual extracts for above experimental conditions were determined with one-way Analysis of Variance (one-way ANOVA, $P< 0.05$) (SAS 9.4 TS1M3) (Bozkurt et al., 2014; Ailavadi et al., 2019).

3. Results:

Various reports indicate that GRAS category compounds have been investigated for their antiviral and antibacterial properties in the food processing industry (Masschalck et al., 2003; Lopez and Belloso, 2006; del Olmo et al., 2017). In the present study, all the three plant extracts, GSE (1 mg/ml), curcumin (0.015 mg/ml) and gingerol (0.1mg/ml) seem to have heat-sensitization effects on HAV and TV based on their D-values with extracts and in PBS. D-values of HAV with and without plant extracts are summarized in Table 2.1. Using the linear

model, D-values of HAV obtained in this study were 9.21±0.24, 2.63±0.13, 1.80±0.08 and 0.67±0.19 min from 56, 60, 65 and 68 ^{o}C respectively, with a z-value of 11.65±0.12 ^{o}C. These values were similar to prior data obtained on the thermal inactivation of HAV in buffer, which were 8.40±0.4 to 0.88±0.11 min from 56 to 72 ^{o}C using the linear model (Bozkurt et al, 2014). HAV when heat-treated with GSE (1mg/ml) showed a significant decrease in D-values at 60 and 65 ^{o}C ($P<0.05$), but no significant difference in the D-values was obtained at 56 ^{o}C and 68 ^{o}C ($P>0.05$). D-values of HAV with 1 mg/ml GSE at 56, 60, 65 and 68 ^{o}C were 8.91±0.12, 1.92±0.17, 1.58±0.12, and 0.63±0.07 min, respectively, with a z-value of 11.54±0.23 ^{o}C. Similarly, for HAV with 0.015 mg/ml curcumin, D-values at 56, 60, 65, and 68^{o}C were 8.12±0.18, 1.78±0.15, 1.43±0.12, 0.55±0.08 min, respectively, with a z-value of 11.53±0.07 ^{o}C. In this particular case, D-values differed significantly at 56, 60 and 65^{o}C ($P<0.05$). For HAV with 0.1mg/ml gingerol, D-values at 56, 60, 65 and 68 ^{o}C were 8.87±0.18, 1.94±0.17, 1.52±0.12, 0.60±0.07 min, respectively, with a z-value of 11.80±0.17 ^{o}C. A similar trend was observed for HAV, where the D-values of HAV with and without extracts were significantly different at 60 and 65 ^{o}C ($P<0.05$). In the case of treatments with plant extracts against HAV, the addition of 0.015 mg/ml curcumin showed higher reduction in D-values followed by gingerol and GSE. No significant difference in the D-values of HAV was observed at 68 ^{o}C for all the extracts ($P>0.05$). This may be because at such high temperature, rate of viral inactivation is rapid and hence it is difficult to obtain a pronounced effect of antiviral activity of extracts at such short time intervals.

The D-values of TV with and without plant extracts are reported in Table 2.2. Using the linear model, D-values of TV in buffer at 52, 54, 56 and 58 ^{o}C were found to be 4.59±0.28,

2.93±0.13, 1.79±0.11, 1.08±0.16 min, respectively, with a z-value of 9.48±0.20 ºC. These values are similar to previously reported thermal inactivation data for TV in cell culture media at 52, 54, 56 and 60 ºC of 4.59±0.02, 2.91±0.01, 1.74±0.41 and 0.58±0.36 min, respectively, with a z-value of 9.09±0.01 ºC (Ailavadi et al., 2019). TV with GSE showed a significant decrease in D-values at 52, 54, 56 and 58 ºC which were 3.82±0.18, 2.11±0.27, 1.23±0.09, 0.80±0.07 min with a z-value of 8.80±0.21 ºC ($P<0.05$). D-values of TV with curcumin at 52, 54, 56 and 58 ºC were 4.32±0.25, 1.98±0.17, 1.33±0.12, 0.62±0.17 min with a z-value of 7.40±0.09ºC. TV with gingerol showed D-values of 4.32±0.25, 1.98±0.17, 1.33±0.12, 0.62±0.17 min at 52, 54, 56 and 58 ºC respectively, with a z-value of 8.38±0.07 ºC, which were significantly lower at 54, 56 and 58 ºC ($P<0.05$) compared to the D-values in PBS.

The D-values ($t_{d=1}$) of HAV and TV with and without plant extracts obtained by the Weibull Model are compared in Table 2.3 (a and b) and 2.5 (a and b) respectively. In PBS, D-values ($t_{d=1}$) of HAV were 10.69, 2.74, 2.12 and 0.78 min which were significantly lowered to 7.58, 2.00, 1.80 and 0.64 min with curcumin (0.015 mg/ml) , 4.72, 2.08, 1.49 and 0.66 min with GSE (1mg/ml) and 7.16, 1.79, 1.37 and 0.73 min with gingerol (0.01 mg/ml) from 56 to 68 ºC respectively ($P<0.05$). Similarly, D-values ($t_{d=1}$) of TV in PBS were 6.20, 2.67, 1.90 and 0.72 min, which decreased to 6.05, 2.57, 1.10 and 0.67 min with curcumin (0.015 mg/ml), 4.73, 2.59, 1.51 and 0.63 min with GSE (1mg/ml) and 4.39, 1.77, 1.76 and 0.79 min with gingerol (0.01 mg/ml)at 52, 54, 56 and 58 ºC, respectively. Therefore, the addition of GSE, curcumin and gingerol at the tested concentrations showed decreased D-values for HAV and TV using both the linear and Weibull model, respectively.

The 6 D-values obtained by the first-order and Weibull model for HAV and TV are compared in Table 2.4 and 2.6 respectively. The 6D-values of HAV using the linear model ranged from 55.26 to 4.02 min in PBS, 53.46 to 3.78 min in GSE (1mg/ml), 48.72 to 3.30 min in curcumin (0.015 mg/ml) and 53.22 to 3.6 min in gingerol (0.01mg/ml). Using the Weibull model ($t_{d=6}$), these values significantly differed to 44.12 to 1.88 min in PBS, 30.70 to 1.73 min in GSE (1mg/ml), 54.50 to 1.60 min in curcumin (0.015 mg/ml) and 42.58 to 1.35 min in gingerol (0.01mg/ml) from 56 to 68 ^{o}C, respectively ($P<0.05$), and similarly for TV these trends followed. The 6 D-values of TV using the linear model ranged from 27.54 to 6.48 min in PBS, 22.92 to 4.8 min in GSE (1mg/ml), 25.92 to 3.72 min in curcumin (0.015 mg/ml) and 24.54 to 4.32 min in gingerol (0.01mg/ml) from 52 to 58 ^{o}C, respectively. Using the Weibull model ($t_{d=6}$), the values significantly differed- 23.24 to 7 min in PBS, 21.77 to 2.27 min in GSE (1mg/ml), 19.28 to 2.39 min in Curcumin (0.015 mg/ml) and 16.06 to 2.03 min in Gingerol (0.01mg/ml) from 52 to 58 ^{o}C, respectively ($P<0.05$).

4. Discussion:

With respect to the antiviral activity of plant extracts, HAV titers were shown to decrease in a dose-dependent manner from ~7 log PFU/ml to 4.61 log PFU/ml after treatment at room temperature or 37 °C with 0.5, 1, and 2 mg/ml GSE for 2 h (Su and D'Souza, 2011). Based on current heat inactivation data, subjecting HAV in suspension (~7 log PFU/ml) to heat at 65 ^{o}C, the same amount of reduction can be obtained in 7.28, 6.69 and 7.11 min with the addition of GSE (1 mg/ml), curcumin (0.015 mg/ml), or gingerol (0.1 mg/ml), respectively, which was higher than heating at 65 ^{o}C alone (6.34 log PFU/ml after 1.5 min) using the linear model. Previously, curcumin at 5 µg/mL was shown to decrease FCV titers by 1.75 log $TCID_{50}$/ml at

room temperature after 30 min (Randazzo et al., 2016) and at 2 mg/ml reduced MNV-1 by 90.43% ±9.13% at 4 ^{o}C after 3 days (Yang et al., 2016). In comparison, the current data of heat-inactivation kinetics with curcumin at 0.015 mg/ml showed a 3 log PFU/ml reduction of TV after 3.26 min at 58 ^{o}C using the linear model. Results of the current study with GSE, curcumin and gingerol corroborate well with earlier studies on the heat inactivation of foodborne bacterial pathogens along with plant extracts and spices (Venkitanarayanan et al., 1998; Juneja et al., 2008; Cui et al., 2011; Bevilacqua et al., 2013). Our data shows that HAV (~7 log PFU/mL) was reduced to 2 log PFU/ml in 3 min after heating with 1mg/ml GSE at 60 ^{o}C, an additional 1 log decrease compared to heating in PBS alone at 60 ^{o}C for 3 min, while heating with curcumin (0.015 mg/ml) and gingerol (0.01mg/ml) reduced the initial titer of HAV to ~ 1.7 log PFU/mL after 3 min at 60 ^{o}C. Similarly, TV (~ 6 log PFU/mL) was reduced to 2.3 log PFU/ml after heating with 1 mg/ml GSE, by 2.5 log PFU/mL with curcumin (0.015 mg/ml) and 2.41 PFU/ml with gingerol (0.01mg/ml) after 3 min at 54 ^{o}C. Therefore, each of these plant extracts seemed to have an additive (if not synergistic) effect on the thermal inactivation of HAV and TV at tested temperatures. However, at a higher inactivation temperature of 68 ^{o}C for HAV, where the rate of inactivation is rapid, this additive effect could not be observed as very short time intervals were tested.

During the study of heat inactivation, there are deviations present in linear survival curves, for example, delayed response of the viruses to thermal treatments. These deviations are important in identifying and studying heat resistance of the microorganisms. (Bozkurt et al., 2015). Therefore, the Weibull model is used which takes into account such deviations (Bozkurt et al., 2014, 2015). The shape factor (β) provides "distribution of individual resistances among

the microbial cells within a population" (van Boekel, 2002). When β< 1, the survivors can potentially be able to adapt to the applied thermal stress and a β>1 indicates that survivors can become increasingly damaged during thermal treatments (Bozkurt et al., 2015; Ailavadi et al., 2019). In the present study, the Weibull shape factor (β) ranges for HAV with and without plant extracts from 56 to 68 ^{o}C were 0.049 to 2.471, whereas, the Weibull shape factor (β) ranges for TV with and without plant extracts from 52 to 58 ^{o}C were 0.030 to 1.890. For the highest treatment temperature of 68 ^{o}C (in case of HAV) and 58 ^{o}C (in case of TV), β was >1 (downward shoulder), indicating that respective viruses were increasingly damaged at such high temperatures. These results were consistent with previous studies for HAV and TV (Bozkurt et al., 2014; Ailavadi et al., 2019).

Using the linear model, D-values of HAV from 56 to 68 ^{o}C obtained in this study were 9.21±0.24 to 0.67±0.19 min, 8.12±0.18 to 0.55±0.08 min, 8.91±0.12 to 0.63±0.07 min and 8.87±0.18 to 0.60±0.07 min in PBS, curcumin (0.015mg/ml), GSE (1 mg/ml) and gingerol (0.01 mg/ml), respectively. However, using the Weibull model, D-values of HAV from 56 to 68 ^{o}C were 10.69 to 0.78 min, 7.58 to 0.64 min, 4.72 to 0.66 min and 7.16 to 0.73 min with PBS, curcumin (0.015mg/ml), GSE (1mg/ml) and gingerol (0.1mg/ml), respectively. This indicates that due to the addition of plant extracts, over-processing can potentially occur if the target goal is a one log reduction of HAV using the first-order linear model rather than the Weibull model when using ~7 log of the virus. In case of TV, D-values obtained from 52 to 58 ^{o}C were 4.59±0.28 to 1.08±0.16 min, 4.32±0.25 to 0.62±0.17 min, 3.82±0.18 to 0.80±0.07 min, 4.09±0.18 to 0.72±0.09 min in PBS, curcumin (0.015 mg/ml), GSE (1 mg/ml) and gingerol (0.01 mg/ml) respectively using linear model. For the tested temperatures of 52 to 58 ^{o}C, D-values of

TV were 6.20 to 0.72 min, 6.05 to 0.67 min, 4.73 to 0.63 min and 4.39 to 0.79 min with PBS, curcumin (0.015 mg/ml), GSE (1 mg/ml) and gingerol (0.1 mg/ml) respectively, using the Weibull model. This indicates that under-processing can potentially occur if the target goal is a one log reduction of TV using the linear first-order model instead of using the Weibull model. The addition of plant extracts decreases D-values of HAV and TV irrespective of the model used.

In order to ensure food safety and validate a thermal process, it is important to determine time-temperature regimes required to obtain 6 logs or 6 D reduction of foodborne pathogen (Aryani et al., 2015). Using the linear model data, the calculated 6D values of HAV in PBS ranged from 55.26 to 4.02 min from 56 to 68 ^{o}C and 6D values of TV in PBS ranged from 27.54 to 6.48 min from 52 to 58 ^{o}C. These values are similar to previously determined 6D values of HAV in PBS at 72 ^{o}C, which was 5.3 min (Schmidt et al., 2016) and 6D values of TV in PBS which were 27.55, 17.28, 10.44 and 3.48 min at 52, 54, 56 and 60 ^{o}C, respectively (Ailavadi et al., 2019). GSE (1mg/ml), curcumin (0.015 mg/ml) and gingerol (0.01 mg/ml) decreased 6D-values of HAV from 56 to 68 ^{o}C. When HAV suspension was heat treated with GSE (1 mg/ml), 6D-values ranged from 53.46 to 3.78 min; 6-D values with curcumin (0.015 mg/ml) ranged from 48.72 to 3.30 min and with gingerol (0.01 mg/m) ranged from 53.22 to 3.6 min. A similar phenomenon is observed in the case of TV at 52, 54, 56 and 58 ^{o}C. 6D-values of TV with GSE (1mg/ml) ranged from 22.92 to 4.8 min; with curcumin (0.015 mg/ml) ranged from 25.92 to 3.72 min and with gingerol (0.01mg/ml) ranged from 24.54 to 4.32 min.

By comparing D-values and 6D-values of HAV and TV with above plant extracts, curcumin (0.015 mg/ml or 15 μg/ml) and GSE (1 mg/ml) seemed to be most heat sensitization

effect on HAV and TV when using the linear model, whereas gingerol (0.01 mg/ml) seemed to have highest heat sensitizing activity using the Weibull model. Based on decreasing 6D-values of HAV and TV, it can be concluded that addition of plant extracts can reduce the time and temperature requirements of a thermal process to ensure food safety. Also, it can be observed that time required to achieve 6 log reduction of HAV and TV with and without plant extracts is much higher using the linear model. Hence, the chance of over-processing is greater if the linear model is used to study inactivation kinetics of HAV and TV with GSE, curcumin and gingerol.

In order to maintain the safety, nutrient value and sensory attributes of food while processing, hurdle technologies could be a better alternative as they decrease time-temperature requirements for food processing (Singh and Shalini, 2016). There continues to be increased interest in plant extracts in the research community and food industry associated with their consumer acceptability due to their GRAS status (Burt, 2004). Although, many plant extracts have already been characterized for their antibacterial activity, information regarding their antiviral properties against foodborne viruses is limited (Li et al., 2013). The current study showed that the use of 1 mg/ml GSE, 0.015 mg/ml curcumin and 0.1 mg/ml gingerol can aid in decreasing the temperature-time parameters needed to inactivate both HAV and TV. These extracts can potentially be used in food systems for validation of these results along with determination of the maintenance of sensory attributes and consumer acceptability. Also, the exact mechanism of viral inactivation in presence of plant extracts and heat remains to be elucidated. In case of non-enveloped viruses, such as HNoV and HAV, inactivation due to heat could occur by loss of the viral protein receptors altering physical and biological properties of the virus or by removal minor part of the capsid protein (VP4) followed by exposure of the viral

RNA and subsequent degradation (Briendl, 1997). During heat and chemical treatments, it can be hypothesized that phytochemicals could irreversibly bind to various components of these viral capsid proteins, cause aggregation or blocking of receptors, which can lead to decreases in viral titers or infectivity. Previous studies have shown synergistic interactions of plant extracts against foodborne bacteria (Pei et al., 2009; Periago and Moezelaar, 2001; Periago et al., 2001). Although, individual extracts (GSE (1 mg/ml), curcumin (0.015 mg/ml) and gingerol (0.1 mg/ml)) show increased effects when combined with heat against HAV and TV, there is no conclusive data to determine the exact mechanism of action and their synergistic or additive properties. Therefore, another aspect for future research with using natural food additives for viral inactivation is studying their interaction with each other as well as their behavior in food matrices.

5. Conclusions:

Due to the rise in incidents of foodborne viral outbreaks, there is a need to determine efficient enteric virus inactivation methods. Use of plant extracts can perhaps maintain food safety and consumer acceptability by decreasing the time and energy requirements during the application of heat inactivation regimes and during food processing. This study showed that decreased time was needed using GSE, curcumin and gingerol for HAV and TV inactivation in PBS within 2-ml glass vials at above tested temperatures. Thus, these extracts can be considered as alternate choices for food processing industries in order to ensure food safety and potentially maintain sensory profiles. However, sensory testing as well as heat inactivation studies after application in food matrices needs to be undertaken.

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Appendix:

Table 2.1: Comparison of D-values of HAV in PBS with GSE (1mg/ml), Curcumin (15 µg/ml) and Gingerol (0.1mg/ml) at various temperatures using the linear model

T (°C)	D-values of HAV in PBS	R^2	Z-value (°C)	D-values of HAV with 1 mg/ml of GSE	R^2	Z-value (°C)	D-values of HAV with 0.015 mg/ml Curcumin	R^2	Z-value (°C)	D-values of HAV with 0.1 mg/ml of Gingerol	R^2	Z-value (°C)
56	9.21±0.24^{A}	0.93	11.65±0.12	8.91±0.12^{A}	0.91	11.54±0.23	8.12±0.18^{B}	0.90	11.53±0.07	8.87±0.18^{B}	0.93	11.80±0.17
60	2.63±0.13^{A}	0.95		1.92±0.17^{B}	0.89		1.78±0.15^{B}	0.89		1.94±0.17^{B}	0.89	
65	1.8±0.08^{A}	0.95		1.58±0.12^{B}	0.92		1.43±0.12^{B}	0.90		1.52±0.12^{B}	0.91	
68	0.67±0.19^{A}	0.90		0.63±0.07^{A}	0.95		0.55±0.08^{A}	0.92		0.60±0.07^{A}	0.91	

Data represents means ± standard deviations of three measurements
Uppercase bold letters when compared across columns denote significant differences between D-values within one temperature for one plant extract ($P<0.05$)

Table 2.2: Comparison of D-values of TV in PBS to D-values of TV with GSE (1mg/ml), Curcumin (15 µg/ml) and Gingerol (0.1mg/ml) at various temperatures using the linear model

T (°C)	D-values of TV in PBS	R^2	Z-value (°C)	D-values of TV with 1 mg/ml of GSE	R^2	Z-value (°C)	D-values of TV with 15 µg/ml Curcumin	R^2	Z-value (°C)	D-values of TV with 0.1 mg/ml of Gingerol	R^2	Z-value (°C)
52	4.59±0.28^{A}	0.90	9.48±0.2	3.82±0.18^{B}	0.92	8.80±0.21	4.32±0.25^{A}	0.92	7.40±0.09	4.09±0.18^{A}	0.91	8.38±0.07
54	2.93±0.23^{A}	0.92		2.11±0.27^{B}	0.90		1.98±0.17^{B}	0.90		1.28±0.27^{B}	0.92	
56	1.79±0.11^{A}	0.91		1.23±0.09^{B}	0.91		1.33±0.12^{B}	0.90		0.97±0.15^{B}	0.93	
58	1.08±0.16^{A}	0.94		0.80±0.07^{B}	0.94		0.62±0.17^{B}	0.91		0.72±0.09^{B}	0.93	

Data represents means ± standard deviations of three measurements
Uppercase bold letters when compared across columns denote significant differences between D-values within one temperature for one plant extract ($P<0.05$)

Table 2.3 (a): Comparison of D-values ($t_{D=1}$) (min) and 6D-values ($t_{D=6}$) (min) of HAV in PBS with GSE (1mg/ml), Curcumin (15 µg/ml) and Gingerol (0.1mg/ml) at various temperatures using Weibull model

T (°C)	HAV+PBS		HAV+ GSE (1 mg/ml)		HAV+ Curcumin (0.015 mg/ml)		HAV+ Gingerol (0.01 mg/ml)	
	$t_{D=1}$	$t_{D=6}$	$t_{D=1}$	$t_{D=6}$	$t_{D=1}$	$t_{D=6}$	$t_{D=1}$	$t_{D=6}$
56	10.69	44.12	4.72	30.70	7.586	54.50	7.164	42.58
60	2.746	11.89	2.089	7.07	2.001	7.352	1.796	7.566
65	2.127	3.985	1.490	2.868	1.806	3.364	1.376	2.765
68	0.781	1.882	0.666	1.734	0.648	1.602	0.731	1.359

Data represents mean of three measurements

Table 2.3 (b): Comparison of scale factor (α) (min) and shape factor (β)of HAV in PBS with GSE (1mg/ml), Curcumin (15 µg/ml) and Gingerol (0.1mg/ml) at various temperatures using Weibull model

T (°C)	**HAV+PBS**			**HAV+ GSE (1 mg/ml)**			**HAV+ Curcumin (0.015 mg/ml)**			**HAV+ Gingerol (0.01 mg/ml)**		
	α	**β**	**R^2**	**α**	**β**	**R^2**	**α**	**β**	**R^2**	**α**	**β**	**R^2**
56	6.943	1.126	0.99	1.988	0.975	0.99	3.045	0.908	0.99	3.129	1.005	0.99
60	1.818	1.223	0.99	1.183	1.468	0.97	1.105	1.223	0.98	0.919	1.246	0.99
65	1.588	2.853	0.89	1.099	2.737	0.82	1.374	2.737	0.86	0.994	2.569	0.84
68	0.519	2.039	0.91	0.427	1.874	0.93	0.405	1.874	0.92	0.548	2.892	0.83

Data represents mean of three measurements

Table 2.4: Comparison of 6D-values (min) of HAV in PBS and plant extracts using first-order kinetics and Weibull distribution at various temperatures

T (ºC)	HAV+PBS		HAV+ GSE (1mg/ml)		HAV+ Curcumin (0.015mg/ml)		HAV+ Gingerol (0.01mg/ml)	
	First-order kinetics (min)	Weibull distribution (min)	First-order kinetics (min)	Weibull distribution (min)	First-order kinetics (min)	Weibull distribution (min)	First-order kinetics (min)	Weibull distribution (min)
56	55.26^{A}	44.12^{B}	53.46^{C}	30.70^{D}	48.72^{E}	54.50^{F}	53.22^{G}	42.58^{H}
60	15.78^{A}	11.89^{B}	11.52^{C}	7.07^{D}	10.68^{E}	7.35^{F}	11.64^{G}	7.56^{H}
65	10.8^{A}	3.985^{B}	9.48^{C}	2.868^{D}	8.58^{E}	3.36^{F}	9.21^{G}	2.76^{H}
68	4.02^{A}	1.88^{B}	3.78^{C}	1.73^{D}	3.3^{E}	1.60^{F}	3.6^{G}	1.35^{H}

Data represents mean deviations of three measurements
Uppercase bold letters when compared across columns denote significant differences between 6 D-values within two models ($P<0.05$)

Table 2.5 (a): Comparison of D-values ($t_{d=1}$) (min) and 6D- values ($t_{d=6}$) (min) of TV in PBS with GSE (1mg/ml), Curcumin (15 µg/ml) and Gingerol (0.1mg/ml) at various temperatures using Weibull model

T (°C)	TV+PBS				TV+ GSE (1mg/ml)				TV+ Curcumin (0.015mg/ml)				TV+ Gingerol (0.01mg/ml)			
	β	$t_{D=1}$ (min)	$t_{D=6}$ (min)	R^2	β	$t_{D=1}$ (min)	$t_{D=6}$ (min)	R^2	β	$t_{D=1}$ (min)	$t_{D=6}$ (min)	R^2	β	$t_{D=1}$ (min)	$t_{D=6}$ (min)	R^2
52	1.901	6.260	23.24	0.92	1.175	4.738	21.77	0.99	1.548	6.056	19.28	0.96	1.076	4.39	16.06	0.99
54	0.881	2.671	19.69	0.96	1.356	2.599	9.740	0.99	1.341	2.577	10.16	0.98	1.135	1.77	8.609	0.99
56	0.871	1.903	13.335	0.99	1.153	1.519	8.609	0.99	1.300	1.709	7.552	0.98	1.337	1.769	6.576	0.98
58	0.787	0.726	7.006	0.98	1.409	0.636	2.270	0.97	1.413	0.672	2.391	0.97	1.908	0.796	2.037	0.92

Data represents mean of three measurements
Uppercase bold letters when compared across columns denote significant differences between 6 D-values within two models ($P<0.05$)

Table 2.5 (b): Comparison of scale factor (α) (min) and shape factor (β)of TV in PBS with GSE (1mg/ml), Curcumin (15 µg/ml) and Gingerol (0.1mg/ml) at various temperatures using Weibull model

T (°C)	TV+PBS			TV+ GSE (1 mg/ml)			TV+ Curcumin (0.015 mg/ml)			TV+ Gingerol (0.01 mg/ml)		
	α	β	R^2	α	β	R^2	α	β	R^2	α	β	R^2
52	4.005	1.901	0.92	2.339	1.175	0.99	3.553	1.548	0.96	2.027	1.076	0.99
54	1.000	0.881	0.96	1.408	1.356	0.99	1.437	1.341	0.98	0.851	1.135	0.99
56	0.657	0.871	0.99	0.737	1.153	0.99	0.984	1.300	0.98	0.948	1.337	0.98
58	0.251	0.787	0.98	0.352	1.409	0.97	0.372	1.413	0.97	0.514	1.908	0.92

Data represents mean of three measurements

Table 2.6: Comparison of 6D-values (min) of TV in PBS and plant extracts using first-order kinetics and Weibull distribution at various temperatures

T (ºC)	TV+PBS		TV+ GSE (1mg/ml)		TV+ Curcumin (0.015mg/ml)		TV+ Gingerol (0.01mg/ml)	
	First-order kinetics (min)	Weibull distribution (min)	First-order kinetics (min)	Weibull distribution (min)	First-order kinetics (min)	Weibull distribution (min)	First-order kinetics (min)	Weibull distribution (min)
52	27.54^{A}	23.24^{B}	22.92^{C}	21.77^{D}	25.92^{E}	19.28^{F}	24.54^{G}	16.06^{H}
54	17.58^{A}	19.69^{B}	12.66^{C}	9.74^{D}	11.88^{E}	10.16^{F}	7.68^{G}	8.60^{H}
56	10.74^{A}	13.33^{B}	7.38^{C}	8.60^{D}	7.98^{E}	7.55^{F}	5.82^{G}	6.57^{H}
58	6.48^{A}	7^{B}	4.8^{C}	2.27^{D}	3.72^{E}	2.39^{F}	4.32^{G}	2.03^{H}

Data represents mean of three measurements
Uppercase bold letters when compared across columns denote significant differences between 6 D-values within two models ($P<0.05$)

CHAPTER III

COMPARISON OF THERMAL INACTIVATION BETWEEN *STAPHYLOCOCCUS CARNOSUS*CS-299 AND CS-300 AS POTENTIAL HEPATITIS A VIRUS SURROGATES

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Running title: *Comparing the thermal resistance of S. carnosus strains CS 299 and CS 300*

Key words: Hepatitis A virus, *Staphylococcus carnosus*, first order model, D-value and Z-value

Abstract:

Traditionally, heat has been the most popular method for inactivation of pathogens as well as spoilage microbes in food to enhance safety and increase shelf-life. Increasing foodborne human viral illness outbreaks have increased research to determine optimal thermal parameters (D- and z-values) for their inactivation. Studies report that hepatitis A virus (HAV) is more resistant to heat ($D_{72°C}$ of 0.9 min in buffer) than known vegetative bacteria. Non-pathogenic surrogates are used to validate heat-inactivation processes. *Staphylococcus carnosus* is a gram-positive, non-pathogenic bacteria used in the food fermentation industry. The purpose of this study was to (1) compare the thermal inactivation (D- values) of two strains of *S. carnosus* (CS-299 and CS-300) as potential HAV surrogates and (2) determine the effect of modified growth conditions (higher temperature, salt, low pH) and as a bacterial lawn, on their thermal resistance. *S. carnosus* CS299 and CS300 grown at 37 °C and 42 °C overnight (~ 7 log CFU/ml) in tryptic soy broth (TSB), TSB with 4% NaCl and TSB at pH 6 were washed, resuspended in phosphate buffered saline (PBS), individual colonies grown as a lawn on Tryptic Soy Agar plate were scrapped, weighed (~9 log CFU/ml) and then added to sterile 2-ml glass vials. Individual vials containing bacteria were heated in a circulating water bath for various time points at 56, 60, 65 and 68 °C, followed by ice-cooling and surface spread-plating on tryptic soy agar, and incubated at 37 °C for 24 to 48 h. Each treatment in duplicate was replicated three times. For *S. carnosus* CS 299 in TSB at 37 °C, D-values ranged from 5.43±0.18 to 0.33±0.05 min from 56 to 68 °C respectively, and these values increased significantly ($P<0.05$) to 6.74±0.27 to 0.44±0.02 min when grown at 42 °C from and further increased in 4% NaCl and as a lawn. D-values of CS 300 grown in TSB at 37 °C were 6.18±0.25 to 0.54±0.12 min from 56 to 68 °C respectively and increased to 7.09±0.15 to

0.59±0.06 when grown at 42 ºC, in 4% NaCl and further increased as a lawn, similar to CS 299. Both *S. carnosus* strains, though CS300 is marginally better, at high titers (at ~8 log CFU/ml) show potential for use as HAV surrogates due to D-value similarities. Combinations of higher growth temperature with salt and low pH may increase thermal resistance of both strains for improved use as validation surrogates.

1. Introduction:

Human enteric viruses are leading cause to various foodborne outbreaks worldwide. Hepatitis A virus (HAV), a foodborne human enteric viral pathogen, is associated with severe disease symptoms that can last for more than a month, with an increasing number of acute gastroenteritis cases reported each year (Scallan et al., 2011; CDC, 2018; Enkirch et al., 2018;Foster et al., 2018; Michaelis et al., 2018; Moon et al., 2018; Doshani et al., 2019; Wooten, 2019). This positive sense-RNA virus is single-stranded, non-enveloped and belongs to the *Picornaviridae* family. It is environmentally stable, survives adverse environmental conditions, resistant to the extreme pH environment of human gastrointestinal tract (Lemon et al., 2018; D'Souza et al., 2007; Bozkurt et al., 2014). Although viruses cannot grow by themselves in or on food products, foodborne illness can result via contamination of food by virus containing fecal material (Atreya et al., 2004). Transmission of HAV is usually occurs via person-to-person, through the fecal-oral route by consumption of contaminated food or water (CDC, 2017). Due to its ease of transmittance and high persistence, it is important to ensure inactivation of foodborne enteric viruses before consumption of food product. Inactivation of foodborne enteric viruses can be achieved through thermal and non-thermal processes. There are various reports on inactivation of HAV and the cultivable human norovirus surrogates, murine norovirus (MNV), feline calicivirus (FCV) and Tulane virus (TV) through thermal processing, high pressure processing and chemical treatments (Sanglay et al., 2011; Keskinen et al., 2011; Espinosa et al, 2012; Bozkurt et al., 2014; DiCaprio et al, 2015; Ailavadi et al., 2019).

Thermal processing is one of the oldest and widely used method to inactivate foodborne pathogens in order to ensure food safety (US FDA, 2019). Thermal inactivation studies of HAV

and HNoV surrogates in phosphate buffer saline (PBS) by the first order linear model and Weibull model showed that HAV had higher resistance to thermal treatments than the tested cultivable human norovirus surrogates, with a D-value of 2.67±0.42 mins at 60 ^{o}C (Bozkurt et al., 2014). This appears to be more resistant than even *Listeria monocytogenes* to heat treatments that has a reported D-value of 0.35±0.03 min at 60 ^{o}C (Monu et al., 2015). Further studies with food matrices including spinach, deli meats and blue mussel homogenates showed that D-values of HAV were 4.55±0.82, 5.9±1.3, 3.25±0.72 min, respectively at 60 ^{o}C (Bozkurt et al., 2014, 2015), indicating a higher degree of resistance of this virus after treatment of various food products.

It is necessary to validate a thermal process which confirms desired microbial inactivation to ensure compliance according to regulatory guidelines and protocols (Aryani et al., 2015). However, a food processing industry cannot use foodborne pathogen in for thermal process validation. Therefore, vegetative bacteria which are not harmful to humans and do not cause food spoilage are preferred (FDA, 2015). As viruses are difficult to cultivate, non-pathogenic, vegetative, non-spore forming bacteria are used as potential surrogates in order to validate thermal processing for foodborne viruses (Schmidt, 2016; Hu et al., 2017)Some key factors of a suitable and ideal surrogate include that it should be non-pathogenic, should not induce food spoilage, should be aerobic, easy to cultivate and enumerate, genetically stable, should possess consistent growth characteristics and should be transmitted by the same route as that of target pathogen (Hu et al., 2017). Also, the thermal resistance of the surrogates must be equivalent to the resistance of target pathogen to ascertain the efficacy of the thermal process (Busta et al., 2003). Bacterial surrogates such as *Escherichia coli* K12 with D-values at 52 to 68

°C ranging from 9.10 min to 0.22 min in liquid egg white (Jin et al., 2008) are not resistant enough to be suitable HAV surrogates. The most commonly tested bacterial surrogate in studies involving pasteurization, *Listeria innocua*, is also not heat resistant enough for validation studies against HAV, that has a D-value 0.34±0.16 min at 70 ^{o}C (Friedly et al.,2008). Another study compared the thermal resistance parameters of *Staphylococcus carnosus* CS 299 that showed D-values ranging from 478.35±78.35 to 0.36 ±0.07 min from 55 to 70 ^{o}C in phosphate buffer saline (PBS) in 2-ml glass vials (Schmidt et al., 2016). According to this data, *S. carnosus* CS 299 has much lower resistance to thermal treatments than that of HAV in buffer. However, $D_{65}{}^{o}{}_{C}$ of CS 299 grown at 40 ^{o}C in spinach was 1.24±0.04 min comparable to $D_{65}{}^{o}{}_{C}$ of HAV in spinach, concluding that *S. carnosus* may be a potential surrogate for HAV (Schmidt et al., 2016, Bozkurt et al., 2014). This study also indicated that increasing growth temperature may impart higher heat resistance to the bacterial surrogate. *S. carnosus*, a bacteria isolated from dry sausage, is an aerobic, gram-positive, non-spore forming and non-motile which displays a broad growth range from 15 ^{o}C to 45 ^{o}C making it suitable for further study as a potential surrogate (Rosenstein et al., 2008). The thermal resistance of a new strain, *S. carnosus* CS 300 has not been explored to-date, that may have higher thermal resistance to serve as a better surrogate of HAV than CS 299. Therefore, objective of this study was to compare thermal resistance of *S. carnosus* CS 299 and CS 300 grown at 37 ^{o}C and at a higher temperature of 42^{o}C. In addition to growth temperature, previous studies show that presence of sodium chloride, acidic conditions and different inoculation methods can impart high thermal resistance to bacteria (Ivanov et al., 2001; Mazzotta et al., 2001; Sharma et al., 2005; Bae and Lee 2010; Hildebrandt et al., 2016). Thus, another

objective of this study was to compare the thermal resistance of *S. carnosus* CS 299 and CS 300 grown at 37 ºC and 42 ºC in 4% NaCl, at pH 6 and as a lawn.

2. Materials and Methods:

2.1 Bacterial surrogates:

Staphylococcus carnosus strains CS 299 and CS 300 obtained as a gift from Bactoferm (Chr. Hansen, Hoersholm, Denmark) were used in this study. Individual strains were grown in Tryptic Soy Broth (TSB) and streaked on Tryptic Soy Agar (TSA) plates at 37ºC and 42ºC. Isolated colonies from individual strains obtained on TSA plates were further grown and maintained at 37 ºC and 42 ºC for 18 h before further sub-culturing as described in previous studies (Schmidt et al., 2016; Monu et al., 2015)

2.2 Growth in TSB containing NaCl, or at pH 6 and as a bacterial lawn:

To investigate and compare thermal resistance of *S. carnosus* CS 299 and CS 300 grown in the presence of 4% NaCl (salt) and low pH, both strains were inoculated and grown in three sets of TSB tubes- TSB at pH 7.4, TSB containing 4% NaCl, TSB with pH adjusted to 6 (using 1N HCl) at 37 ºC and 42 ºC for 18 h. Additionally, to compare the thermal resistance due to different inoculation methodologies, 1 ml culture of both strains grown at 37 ºC and 42 ºC in TSB was evenly spread on TSA plates to obtain uniform lawns after 18 h at the respective temperatures. Cells grown at both temperatures from both strains for all of these conditions were centrifuged at 5000 xg for 2 min and the bacterial pellet was resuspended in 15 ml of sterile phosphate buffered saline (PBS, pH 7.4). These washed cultures in PBS at 7 log CFU/ml were then transferred to sterile 2-ml screw capped glass vials aseptically before heat treatment. For the bacterial lawn,

cells were air dried under BSL-2 hood for 15 min, scrapped off the TSA plates and aseptically added to sterile glass vials.

2.3 Heat treatment:

For heat treatment experiments, a circulating water bath (ISOTEMP 2150, Fischer Scientific) was used after immersion of the 2ml glass vials containing bacteria as reported earlier (Bozkurt et al., 2014; Ailavadi et al., 2019). Briefly, thermocouple probes (MMS3000, Commtest Instruments) were immersed in such a way that one probe measured the temperature of the circulating water and second probe is at geometric center of a sterile vial containing PBS to record temperature inside the vial. Time taken for the temperature of the vial containing PBS (representing the bacterial suspension) to come-up to that of circulating water (come-up time) was recorded for every experiment performed. Heat inactivation treatment was performed at 56, 60, 65 and 68 ^{o}C for a set of time points. After completion of each time point treatment, in order to facilitate stopping of further reactions, heat treated vials were cooled in an ice-bath immediately, before further performing ten-fold serial dilutions for enumeration (Bozkurt et al., 2014; Schmidt et al., 2016; Ailavadi et al., 2019).

2.4 Enumeration of survivors:

Vials containing bacteria after treatments were surface rinsed with 70% ethanol before enumeration as reported earlier (Bozkurt et al., 2014; Ailavadi et al., 2019). One-ml of heat inactivated bacterial suspension for each time point was ten-fold serially diluted in PBS and survivors were plated on TSA and further incubated for 24 to 48 h at 37 ^{o}C. Thermal inactivation was repeated three times for each temperature and assayed in duplicate.

2.5 Statistical Analysis:

Recovered counts of bacteria from each experiment carried out three times that were assayed in duplicate were statistically analyzed. Colony counts were converted into logarithmic values (log_{10} CFU/mL), to calculate the D-values and z-values (MS Excel, Version 2017). Significant differences of D-values between the two strains grown at two different temperatures for above experimental conditions were determined with one-way Analysis of Variance (one-way ANOVA, $P< 0.05$) (SAS 9.4 TS1M3).

3. Results:

Table 3.1 shows the comparison of the D-values of *S. carnosus* CS 299 and CS 300 grown in TSB at pH 7.4, TSB with 4% NaCl, and TSB at pH 6 at 37 ^{o}C using the linear model. The D-values of *S. carnosus* CS 299 in TSB at 37 ^{o}C ranged from 5.43±0.18 to 0.33±0.05 min from 56 to 68 ^{o}C, while CS 300 had significantly higher D-values than CS 299 which ranged from 6.18±0.25 to 0.54±0.12 min from 56 to 68 ^{o}C ($P<0.05$). In the presence of 4% NaCl (a_w= 0.97), D-values of CS 299 grown at 37 ^{o}C, ranged from 6.74±0.27 to 0.44±0.02 min and that of CS 300 ranged from 8.02±0.19 to 0.48±0.01 min from 56 to 68 ^{o}C. Salt (4% NaCl) imparted significantly higher resistance to both the strains resulting in higher D-values. However, there was no significant difference between D-values of CS 299 and CS 300 in the presence of salt at 37 ^{o}C ($P>0.05$). A similar phenomenon was observed in the case of growth at pH 6. D-values of CS 299 in pH 6 ranged from 6.30±0.13 to 0.45±0.01 min from 56 to 68 ^{o}C and D-values of CS 300 ranged from 7.71±0.19 to 0.44±0.02 min from 56 to 68 ^{o}C. Decreasing the pH of the growth media imparted a significant degree of thermal resistance ($P<0.05$) but within strains, the difference in D-values was not significant ($P>0.05$).

Table 3.2 describes the D-values of both strains exposed to the same conditions of low pH and high salt at a higher growth temperature of 42 ºC. D-values at 56 to 68 ºC for CS 299 at 42 ºC in TSB ranged from 6.96±0.37 to 0.5±0.05 min, while D-values for CS 300 ranged from 7.29±0.15 to 0.59±0.06 min which are significantly higher ($P<0.05$) than that of CS 299. In the presence of 4% NaCl, D-values of CS299 and CS 300 ranged from 7.24±0.1 to 0.50±0.07 min and 8.13±0.19 to 0.68±0.14 min, respectively from 56 to 68 ºC. For growth at pH 6, similar trends in D-values were observed where D-values at 56 to 68 ºC for CS 299 and CS 300 ranged from 7.53±0.23 to 0.48±0.01 min and 7.68±0.12 to 0.49±0.01 min, respectively, and were significantly higher ($P<0.05$) than that of CS 299 and CS 300 grown in TSB at pH 7.4. Tables 3.3 and 3.4 summarize the effect of growing individual strains at various combination of conditions-higher temperature with salt and higher temperature at low pH. It can be observed from D-values that 4% NaCl combined with a higher temperature exert maximum thermal resistance to both the strains, especially CS 300 which have consistently higher D-values. D-values for growth in 4% salt at 42 ºC for CS 299 were 8.54±0.33, 6.41±0.15,1.59±0.08 and 0.89±0.02 min for temperatures of 56, 60, 65and 68 ºC, respectively, and for CS 300 grown under the same conditions were8.58±0.33, 6.52±0.06, 1.62±0.19 and 0.91±0.03 min for the same tested temperatures.

Table 3.5 describes the D-values of CS 299 and CS 300 lawns when grown at 42 ºC. The D-values for CS 299 lawns at 42ºC were 7.14±0.36, 6.57±0.25, 1.25±0.06, 0.69±0.01 min, and that of CS 300 were 7.7±0.27, 7.23±0.29, 1.49±0.11, 0.70±0.15 min at 56, 60, 65 and 68 ºC, respectively. Thus, inoculating cells directly from lawns instead of pelleting from a liquid media may play a role in imparting higher thermal resistance to both the strains. D-values of CS 300 at

56, 60 and 65 ^{o}C in this case were significantly higher ($P<0.05$) than CS 299 suggesting that CS 300 could be preferred over CS 299 as a HAV surrogate for thermal inactivation validation studies at these temperatures.

4. Discussion:

There are several studies which indicate that increasing growth temperature subject bacteria to a certain degree of stress during their growth which is instrumental in increasing their thermal resistance. In the presence of heat, some intra-cellular proteins are denatured which triggers the heat-shock response in the cell resulting in production of heat-shock proteins (hsp) (Mackey and Derrick, 1990). These hsps bind to exposed hydrophobic regions of denatured proteins in an ATP dependent manner and stabilize them by refolding or by disaggregating clumps of thermal denatured proteins (Mackey and Derrick, 1990). These events lead to an increase in heat resistance of the bacterial cell. In this study, D-values of *S. carnosus* CS 299 and CS 300 grown at 37 ^{o}C were 5.43±0.18to 0.33±0.05 min and 6.18±0.25 to 0.54±0.12 min from 56 to 68 ^{o}C, respectively. These values are much lower than previously reported D-values of HAV, which are 20.6±0.43 to 0.88±0.11 from 56 to 72 ^{o}C in PBS (Bozkurt et al., 2014). By increasing the growth temperature of the strains to 42 ^{o}C, D-values slightly increased to 6.96±0.37 to 0.5±0.05 min and 7.29±0.15 to 0.59±0.06 min from 56 to 68 ^{o}C for CS 299 and CS 300, respectively but were lesser than the D-values of HAV at the same temperatures. Addition of salt (4% NaCl) in growth media seemed to have an increased thermal resistance effect of both strains. D-values of CS 299 and CS 300 at 37 ^{o}C in 4% NaCl were 6.74±0.27 to 0.44±0.02 min and 8.02±0.19 to 0.48±0.01 min from 56 to 68 ^{o}C, respectively whereas, when grown at 42 ^{o}C in 4% NaCl, D-values of both strains further increased to 7.24±0.1 to 0.50±0.07 min for CS 299 and 8.13±0.19 to 0.68±0.14

min for CS 300 from 56 to 68 ºC. Comparing D-values of strains in the above altered growth conditions to that of HAV, $D_{65}{}^{o}{}_{C}$ of CS 300 at 42 ºC in 4% NaCl was found to be 1.43±0.11 min which is comparable to $D_{65}{}^{o}{}_{C}$ of HAV (1.73±0.98 min) in buffer (Bozkurt et al., 2014). It has been reported that increases in growth temperature contributes to higher thermal resistance of bacteria (Cebrian et al., 2008; Alvarez-Ordonez et al., 2008; Schmidt, 2016). When *Escherichia coli* W3110 (6 log CFU/mL)was grown at 42 °C, 37 °C, 30 °C, 20 °C or 10 °C for 36, 24, 48, 72 and 148 h respectively and subjected to heat treatment at 57·5 °C for 6 min, D-value at 57·5 °C of *E. coli* (6 log CFU/mL) grown at 42 ºC was 3.70±0.09 min, six-fold higher than that corresponding to cells grown at 10 ºC which was 0.58±0.00 min (Cebrian et al., 2008). In another study, D-value of *S.* Typhimurium (5 log CFU/ml) at 58 ºC increased from 0.04±0.005 min when grown at 10 ºC to 0.46±0.08 min at a growth temperature of 45 ºC (Álvarez-Ordóñez et al., 2008). In addition to above studies, the D-value of *L. monocytogenes* (8 log CFU/ml) grown at 42.8 ºC was 3.7±0.2 min, whereas when grown at 37 ºC the D-value was 1.5±0.00 min after treatment at 60 ºC, indicating that the thermal resistance of the cells increases when grown at a higher temperature (Rowan and Anderson, 1998). It was also observed that increasing growth temperature from 32 ºC to 40 ºC of *S. carnosus* CS 299 changed the D-value from 1.59±0.20 min to 3.13±0.48 min at 65 ºC (Schmidt et al., 2016). In the present study, D-values of CS 299 and CS 300 grown at 42 ºC ranged from 6.96±0.37 to 0.50±0.05 min and 7.09±0.15 to 0.42±0.05 min from 56 to 68 ºC, respectively which are higher than D-values of CS 299 and CS 300 grown at 37 ºC, ranging from 5.43±0.18 to 0.33±0.05 min and 6.18±0.25 to 0.54±0.12 min from 56 to 68 ºC, respectively. These results indicate that increasing growth temperature may induce thermotolerance in these bacteria.

Another alteration in growth conditions to determine heat resistance was the decrease in the pH of growth media from 7.4 to 6.0. D-values of CS 299 and CS 300 grown at 37 ^{o}C in pH 6 were 6.30±0.13 to 0.45±0.01 min and 7.71±0.19 to 44±0.02min; and when grown at 42 ^{o}C in pH 6, D-values of CS 299 and CS 300 were 7.53±0.23 to 0.48±0.01 min and 7.68±0.12 to 0.49±0.01 min, respectively. Lowering pH did not seem to have an effect in increasing the heat resistance of the strains as the D-values of both strains at pH 6 at all temperatures did not differ significantly with the strains grown at pH 7.4 The low pH of growth media is reported to alter the fatty acid composition of the bacterial membranes which may lead to better thermal resistance (Trček et al., 2015). In addition to studies conducted on spore-forming foodborne pathogenic bacteria, *Clostridium* and *Bacillus*, increase in heat resistance through acid adaptation of Gram-negative bacteria including *E. coli*, *Salmonella* and Gram-positive *L. monocytogenes* was investigated earlier (Juneja and Eblen, 1999, Álvarez-Ordóñez et al., 2008, Mazzotta et al., 2001). The $D_{60}{}^{o}{}_{C}$ of *E. coli* in orange juice (OJ) was shown to significantly increase from 1.1±0.35 to 1.7±0.34 min when acid adapted in TSB at pH 5. Similar results were observed for $D_{60}{}^{o}{}_{C}$ of *L. monocytogenes* in orange juice (OJ) where acid adaptation of pH 5 increased $D_{60}{}^{o}{}_{C}$ from 0.21±0.02 to 0.38±0.09 min (Mazzotta et al., 2001).

Salt is known to cause a decrease in water-activity (a_w) of the growth media, which is another factor that can result in heat resistance. When extra-cellular a_w is lower than optimum growth conditions, bacterial cells are forced to produce hsps in order to prevent plasmolysis (Sperber, 1983). These proteins accumulate in cells and indirectly provide protection to bacteria against harsh or lethal environments, which in this case, is high processing temperature, thereby increasing the D-value of bacteria. The results with CS 299 and CS 300 grown in TSB with 4%

NaCl are consistent with some of the previous studies of *L. monocytogenes* which reported an increase in the D-value from 0.4 min using 0.90 M (5.25% w/v) NaCl to 9.53 min in 1.5 M (8.76% w/v) NaCl when treated at 60 ^{o}C (Doyle et al., 2001). Similar results were observed for *E. coli* O157:H7 and *S.* Enteritidis with a reported increase in D-values from 0.5 min to 2 min and 2.5 min to 8 min, respectively at 59.5 ^{o}C when the NaCl concentration was increased from 0.5%w/w to 8.5% w/w (Blackburn et al., 1997). Since these studies used higher concentrations of NaCl than used in this study, further experiments with higher salt concentrations may also provide information on surrogates with increased thermal resistance. Another method of reducing water-activity is to create low moisture environments for bacterial growth. One study demonstrated that, $D_{60^{o}C\text{-}62^{o}C}$ of *Salmonella weltevreden* inoculated in flour was 875 min at an initial a_w of 0.4 and $D_{63^{o}C\text{-}65^{o}C}$ was 29 min at an initial a_w of 0.5 (Archer et al, 1998). In the case of *Salmonella, L. monocytogenes* and *E. faecium*, $D_{90^{o}C}$ at a_w of 0.65 were 1.53±0.07 min, 1.54±0.01 min, 4.56±0.05 min, respectively which increased to 17.76±1.54 min, 1.68±0.10 min and 23.40±1.53 min, respectively when the a_w was reduced to 0.38 (Rachon et al., 2016). Similar results were observed for *S.* Enteritidis and *E. faecium* treated at 80 ^{o}C on silicon dioxide carriers. $D_{80^{o}C}$ of *S.* Enteritidis and *E. faecium* at a_w 0.70 were 1.80±0.12 min and 3.81±0.11 min, respectively which sharply increased to 159.31± 5.77 min and 281.78±5.78 min when the a_w was reduced to 0.11 (Liu et al., 2018). Results described in the present study are consistent with these above data. D-values of CS 299 and CS 300 grown at 42 ^{o}C as a lawn (a_w= 0.48) on TSA are 7.14±0.36, 6.57±0.25, 1.25±0.06, 0.69±0.01 min and 7.7±0.27, 7.23±0.29, 1.49±0.11, 0.70±0.15 min at 56, 60, 65 and 68 ^{o}C, respectively which are significantly higher than D-values of CS 299 and CS 300 in grown at 42 ^{o}C in TSB which were 6.96±0.37, 5.26±0.04, 0.94±0.10, 0.5±0.05

min and 7.29±0.15, 6.27±0.04, 1.09±0.02, 0.42±0.05 min, respectively from 56 to 68 ^{o}C ($P<0.05$).

Taking into account the changes in D-values and increases in thermal resistance of CS 299 and CS 300, it can be concluded that CS 300 grown at 42 ^{o}C in 4% NaCl has the highest D-values among all tested temperatures. Thus, our study shows that combination of salt and higher growth temperature had an additive effect that increased the thermal resistance of both the tested strains. For the non-pathogenic *S. carnosus* CS 299 and CS 300, the exact mechanism of heat resistance is still unclear. It is important to note that these studies were conducted in PBS and that their behavior and thermal resistance may vary in presence of different food matrices that need further investigation.

For process validation, 6D-values have been used to evaluate the thermal inactivation potential of various time-temperature combinations in food processing. (Aryani et al., 2015). These values determine the performance criteria of a thermal process to ensure food safety (van Schothorst, 1998). In this particular experiment, using the linear model, the 6 D-values of CS 300 grown at 42 ^{o}C in 4% NaCl, which showed the highest D-values among the tested conditions were compared with the 6 D-values of HAV and reported in Table 6. Thus, at 65 ^{o}C, 6D-value of CS 300 grown at 42 ^{o}C in 4% NaCl was 8.58 min, which is somewhat close to the 6D-value of HAV at 65 ^{o}C, which is 10.38 min. Using this data, about 7.25 log CFU/ml of CS 300 grown at above mentioned condition would be required to achieve a reduction equivalent to 6 log HAV in order to validate a thermal process. Based on 6-D projections, CS 300 grown at 42 ^{o}C in 4% NaCl, could be potentially used as a surrogate for foodborne bacteria including *L.*

monocytogenes, *S. enterica*, *E. coli* O157:H7 (Monu et al., 2015) as well as HAV using higher bacterial populations of 8 log CFU/ml.

5. **Conclusions:**

Overall, this study provided data on the comparison of the thermal resistance of *S. carnosus* CS 299 and CS 300 and suggests that CS 300 grown at 42 ºC in 4% NaCl is has improved heat resistance compared to CS 299 for use in heat-inactivation validation studies, that showed a D-value of 1.43±0.11min at 65 ºC close to the D-value of HAV of 1.73±0.98 min at 65 ºC (Bozkurt et al, 2014).

Furthermore, external stressors such as higher growth temperature, presence of salt and decreasing pH can result in an increase in thermal resistance of these bacterial strains. Further studies combining these factors to determine additional increases in thermal resistance for further inoculation studies in various food products are needed.

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Appendix:

Table 3.1: D- values (min) of *S. carnosus* CS299 and CS 300 grown at 37 °C in TSB, TSB containing 4% NaCl and at pH 6

T	TSB				TSB with 4% NaCl				TSB at pH6			
(°C)	CS 299	R^2	CS 300	R^2	CS 299	R^2	CS 300	R^2	CS 299	R^2	CS 300	R^2
56	5.43 ± 0.18^{C}	0.90	6.18 ± 0.25^{B}	0.91	6.74 ± 0.27^{B}	0.90	8.02 ± 0.19^{A}	0.92	6.30 ± 0.13^{B}	0.91	7.71 ± 0.19^{A}	0.93
60	4.32 ± 0.26^{B}	0.92	5.21 ± 0.18^{A}	0.95	5.03 ± 0.11^{A}	0.93	5.36 ± 0.29^{A}	0.93	4.79 ± 0.11^{AB}	0.97	5.29 ± 0.24^{A}	0.90
65	0.87 ± 0.04^{D}	0.97	0.93 ± 0.09^{CD}	0.95	1.19 ± 0.04^{AB}	0.95	1.40 ± 0.07^{A}	0.95	1.14 ± 0.08^{BC}	0.95	1.40 ± 0.11^{A}	0.91
68	0.33 ± 0.05^{B}	0.90	0.54 ± 0.12^{A}	0.96	0.44 ± 0.02^{AB}	0.91	0.48 ± 0.01^{AB}	0.91	0.45 ± 0.01^{AB}	0.93	0.44 ± 0.02^{AB}	0.91

Data represents means ± standard deviations of three measurements

Upper case letters across columns denote significant differences between D-values of both strains subjected to different conditions at fixed temperature ($P<0.05$)

Table 3.2: D-values (min) of *S. carnosus* CS 299 and CS 300 grown at 42 ºC in TSB, TSB containing 4% NaCl and at pH 6

T	TSB				TSB with 4% NaCl				TSB at pH6			
(ºC)	CS 299	R^2	CS 300	R^2	CS 299	R^2	CS 300	R^2	CS 299	R^2	CS 300	R^2
56	6.96 ± 0.37^{C}	0.92	7.29 ± 0.15^{BC}	0.94	7.24 ± 0.17^{BC}	0.91	8.13 ± 0.19^{A}	0.92	7.53 ± 0.23^{BC}	0.92	7.68 ± 0.12^{AB}	0.92
60	5.26 ± 0.04^{BC}	0.91	6.66 ± 0.23^{A}	0.95	5.11 ± 0.18^{C}	0.93	5.71 ± 0.13^{B}	0.93	4.93 ± 0.24^{BC}	0.94	5.46 ± 0.09^{BC}	0.91
65	0.94 ± 0.10^{D}	0.93	1.04 ± 0.05^{CD}	0.95	1.29 ± 0.04^{AB}	0.95	1.43 ± 0.11^{A}	0.95	1.17 ± 0.01^{BC}	0.90	1.34 ± 0.07^{AB}	0.91
68	0.5 ± 0.05^{AB}	0.94	0.59 ± 0.06^{AB}	0.96	0.50 ± 0.07^{AB}	0.91	0.68 ± 0.14^{A}	0.91	0.48 ± 0.01^{B}	0.91	0.49 ± 0.01^{AB}	0.90

Data represents means ± standard deviations of three measurements

Upper case letters across columns denote significant differences between D-values of both strains subjected to different conditions at fixed temperature ($P<0.05$)

Table 3.3: D-values (min) of *S. carnosus* CS 299 grown in TSB, 4% NaCl and pH 6 at 37 ºC and 42 ºC

T (ºC)	CS 299 at 37ºC in TSB	R^2	CS 299 at 42ºC in TSB	R^2	CS 299at 37ºCin 4% NaCl	R^2	CS 299 at 37º C in pH 6	R^2	CS 299 at 42ºC in 4% NaCl	R^2	CS 299 at 42ºC in pH 6	R^2
56	5.43 ± 0.18^{D}	0.90	6.96 ± 0.37^{A}	0.92	6.74 ± 0.27^{BC}	0.90	6.30 ± 0.13^{A}	0.91	7.24 ± 0.17^{C}	0.91	7.53 ± 0.23^{B}	0.92
60	4.32 ± 0.26^{B}	0.92	5.26 ± 0.04^{A}	0.91	5.03 ± 0.11^{A}	0.93	4.79 ± 0.11^{A}	0.97	5.11 ± 0.18^{A}	0.93	4.93 ± 0.24^{A}	0.94
65	0.87 ± 0.04^{B}	0.97	0.94 ± 0.10^{B}	0.93	1.19 ± 0.04^{A}	0.95	1.14 ± 0.08^{A}	0.95	1.29 ± 0.04^{A}	0.95	1.17 ± 0.01^{A}	0.90
68	0.33 ± 0.05^{B}	0.90	0.5 ± 0.05^{A}	0.94	0.44 ± 0.02^{A}	0.91	0.45 ± 0.01^{A}	0.93	0.50 ± 0.07^{A}	0.91	0.48 ± 0.01^{A}	0.91

Data represents means ± standard deviations of three measurements

Upper case letters across columns denote significant differences between D-values of one strain subjected to different conditions at two different temperatures ($P<0.05$)

Table 3.4: D-values (min) of *S. carnosus* CS 300 grown in TSB, 4% NaCl and pH 6 at 37 ºC and 42 ºC

T (ºC)	CS 300 at 37ºC in TSB	R^2	CS 300 at 42ºC in TSB	R^2	CS 300 at 37ºC in 4% NaCl	R^2	CS 300 at 37ºC in pH 6	R^2	CS 300 at 42ºC in 4% NaCl	R^2	CS 300 at 42ºC in pH 6	R^2
56	6.18 ± 0.25^{C}	0.91	7.29 ± 0.15^{B}	0.94	8.02 ± 0.19^{A}	0.92	7.71 ± 0.19^{A}	0.92	8.13 ± 0.19^{A}	0.92	7.68 ± 0.12^{A}	0.92
60	5.21 ± 0.18^{B}	0.95	6.66 ± 0.23^{A}	0.95	5.36 ± 0.29^{A}	0.93	5.29 ± 0.24^{A}	0.91	5.71 ± 0.13^{A}	0.93	5.46 ± 0.09^{A}	0.91
65	0.93 ± 0.09^{B}	0.95	1.04 ± 0.05^{B}	0.95	1.40 ± 0.07^{A}	0.95	1.40 ± 0.11^{A}	0.91	1.43 ± 0.11^{A}	0.95	1.34 ± 0.07^{A}	0.91
68	0.54 ± 0.12^{A}	0.96	0.59 ± 0.06^{A}	0.96	0.48 ± 0.01^{A}	0.91	0.44 ± 0.02^{A}	0.90	0.68 ± 0.14^{B}	0.91	0.49 ± 0.01^{A}	0.90

Data represents means ± standard deviations of three measurements

Upper case letters across columns denote significant differences between D-values of one strain subjected to different conditions at two different temperatures ($P<0.05$)

Table 3.5: D-values (min) of *S. carnosus* CS 299 and CS 300 grown as a lawn at 42°C

T (°C)	CS 299	R^2	CS 300	R^2
56	7.14±0.36^{A}	0.94	7.7±0.27^{A}	0.97
60	6.57±0.25^{A}	0.92	7.23±0.29^{B}	0.93
65	1.25±0.06^{A}	0.90	1.49±0.11^{B}	0.90
68	0.69±0.01^{A}	0.93	0.70±0.15^{A}	0.96

Data represents means ± standard deviations of three measurements.
Upper case letters across columns denote significant differences between D-values of both strains at fixed temperature ($P<0.05$)

Table 3.6: Comparison of 6-D values (min) of the CS 300 grown at 42 ºC in 4% NaCl, as lawn with HAV in phosphate buffer saline (PBS) using first-order model

T (ºC)	D-values of CS 300 in 4% NaCl	6D-values of CS 300 in 4% NaCl	D-values of CS 300 as lawn	6D-values of CS 300 as lawn	D-values of HAV in PBS	6D-values of HAV in PBS
56	8.13±0.19	48.78	7.7±0.27	46.2	20.6±0.43	123.6
60	5.71±0.13	34.26	7.23±0.29	43.38	2.67±0.43	16.02
65	1.43±0.11	8.58	1.49±0.11	8.94	1.73±0.98	10.38
68	0.68±0.14	4.08	0.70±0.15	4.2	0.88±0.11	5.3

Data represents means ± standard deviations of three measurements

CHAPTER IV

CONCLUSIONS

Foodborne enteric viruses continue to cause outbreaks that increases the economic burden worldwide. The incidence of foodborne viral illnesses continues to be on the rise, yet their detection and elimination strategies are limited. Appropriate thermal processing parameters are needed for effective control of viral contamination to prevent outbreaks. The knowledge of specific time-temperature requirements to inactivate foodborne viruses and for validation during industrial thermal processes is critical.

Heat sensitization of foodborne viruses by plant extracts in foods need to be understood. The present study investigated the heat sensitization potential of grape seed extract (GSE) (1 mg/ml), curcumin (0.015 mg/ml) and gingerol (0.1 mg/ml) against hepatitis A virus (HAV) and Tulane virus (TV), a cultivable human norovirus surrogate. D-values for TV in PBS (4.59±0.28 to 1.08±0.16 min) significantly decreased to 3.82±0.18 to 0.80±0.07 min with 1 mg/ml GSE, 4.32±0.25 to 0.62±0.17 min with 0.015 mg/ml curcumin, 4.09±0.18 to 0.72±0.09 min with 0.1 mg/ml gingerol using the linear model ($P<0.05$).Similar trends were also observed for HAV. The heat-treatment time needed to obtain a 6-log reduction of HAV and TV was lowered by the addition of plant extracts where the Weibull model provided significantly lower 6 D-values than the linear model for all the extracts in most instances ($P<0.05$). Therefore, addition of plant extracts could lower the time-temperature requirements for enteric viral inactivation. Further studies could focus on determining the synergistic effects of gingerol and curcumin in combination and their interaction with different food matrices on the thermal inactivation kinetics of human enteric viruses.

Even though thermal inactivation kinetics (D- and z-values) of HAV are known, for validation studies finding a non-pathogenic, vegetative, bacterial surrogate with similar heat

resistance profiles as HAV for thermal processing remains challenging. *Staphylococcus carnosus* CS 299 and CS 300 could be used as a potential HAV surrogates by increasing their thermal resistance. This study showed that changing growth conditions for CS 299 and CS 300 can increase their thermal resistance. D-values for CS 299 and CS 300 when grown at 42 ^{o}C in Tryptic Soy broth (TSB) were significantly higher than D-values of CS 299 and CS 300 grown at 37 ^{o}C ($P<0.05$). Growth at pH 6 at 37 ^{o}C and 42 ^{o}C did not show a significant increase in thermal resistance compared to growth at pH 6 ($P>0.05$), but the addition of salt (4% NaCl) at higher growth temperature of 42 ^{o}C significantly increased the thermal resistance of both strains ($P<0.05$). CS 300 grown at 42 ^{o}C in 4% NaCl had a D-value of 1.43±0.11 min at 65 ^{o}C which was relatively comparable to the D-value of HAV at 65 ^{o}C (1.73 min) in buffer (Bozkurt et al., 2014). Also, 6 D-values (linear model) of CS 300 grown at 42 ^{o}C in 4% NaCl showed the highest D-values of 8.58 min among the tested conditions, somewhat close to 6 D-values of HAV at 65 ^{o}C in buffer, which is 10.38 min (Bozkurt et al., 2014). Using this data, 7.25 log CFU/mL of CS 300 grown at these-described conditions would be required to achieve equivalent 6 log HAV reduction in order to validate a thermal process. Further research could look into increasing thermal resistances of CS 299 and CS 300 by using combinations of increased salt concentration, decreased pH and comparing thermal inactivation kinetics of adapted strains to HAV in food products.

VITA

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