# Intercellular Production of Hydrogen Peroxide in *Solanum lycopersicum*: An Immune Response to *Salmonella* Infection

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# A B S T R A C T

Produce associated gastroenteritis in recent years has become more associated with fruits and vegetables rather than raw meat and dairy, with thousands of deaths per year recorded. Outbreaks of *Salmonella* have been reported from a multitude of vegetables such as lettuce, spinach and tomatoes. Many of these outbreaks are the result of infection via non-typhoid *Salmonella* species such as *Salmonella enterica* sv. Typhimurium. The ecology of *Salmonella* as a human pathogen via vegetable hosts is increasingly being studied, however much is still unknown. Studies have shown that *Salmonella* may proliferate in both the rhizosphere and phyllosphere of the tomato plant *Solanum lycopersiscum.* In tomatoes, *Salmonella* has varying levels of proliferation success depending on the variety of tomato in question. Previous studies had shown that tomatoes of the cherry variety were significantly more resistant to *Salmonella* proliferation than larger varieties. *S. lycopersicum* var. *cerasiforme* and var. *alicante* were inoculated with *Salmonella enterica* (LT2) sv. Typhimurium and allowed to incubate for 72 hours. Following this, tomatoes were homogenised and samples plated on XLD. This study found that *Salmonella* ability to proliferate in var. *cerasiforme* was significantly different ($\alpha=0.05$) when compared to var. *alicante.* With *Salmonella* proliferating up to a 1 log more in var. *alicante* than in var. *cerasiforme.* This study suggested that this difference was due to an innate difference in immune response efficacy between var. *cerasiforme* and var. *alicante.* Studies regarding plant immune responses have shown that production of hydrogen peroxide is a common response to microbial attack. Using a novel methodology, this study extracted the intercellular fluid from the tomato fruit in both var. *alicante* and var. *cerasiforme.* Analysis via fluorometric quantification found that var. *cerasiforme* contained significantly higher amounts of hydrogen peroxide in its intercellular fluid than var. *alicante.* These data suggest that the inherent and increased resistance to *Salmonella* proliferation in var. *cerasiforme* compared to var. *alicante* is at least in part due to its increased levels of hydrogen peroxide in its intercellular fluid.

## A C K N O W L E D G E M E N T S

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# 1. INTRODUCTION

## 1.1 Foodborne Illness and Vegetable Produce

Foodborne illness and disease has been a continuing and persisting issue for many years. Each year, food borne pathogens cause thousands of deaths and many more illnesses in the USA alone (Lutter, 1999). As the populace grows so does the demand for food. Projections from The Food and Agriculture Organisation (FAO) say that by 2050 the global demand for food will have doubled due to economic and population growth (Population Action International, 2012). There is an ever more important need to feed populations and communities, but to also ensure that the food grown and cultivated is safe and do not cause disease.

Salmonellosis (the disease causes by infection of *Salmonella enterica* sv. Typhimurium.) is often associated with the meat industry, specifically from poultry meat and eggs. In many cases *Salmonella* spp. has been isolated from raw chicken as well as egg shells. In addition, case incidents of salmonellosis have been noted across the globe from China to Australia (Fearnley et al., 2011; Qiao et al., 2017; Sukumaran et al., 2015).

However, the public are often less familiar with the association between salmonellosis and fresh vegetables, despite the fact consumers are more likely to encounter pathogenic enteric bacteria through fresh vegetable produce than meats or other previously noted sources (Centers for Disease Control and Prevention, 2019). Outbreaks of salmonellosis has been associated with produce such as lettuce, spinach, cantaloupe and tomatoes (Barak et al., 2008; Centers for Disease Control and Prevention (CDC), 2007, 2005; Cooley et al., 2007). Of note, was an outbreak of salmonellosis from Roma tomatoes in 2006 that had a reported 183 cases, 12% of which resulted in hospitalisations (Centers for Disease Control and Prevention, 2007). This particular outbreak showed cases of salmonellosis in sixteen different states within the United States, however the infected tomatoes had originated from a single source in Florida. As with many infection diseases, modern travel and distribution means that a single source infection may affect individuals over a large distance. More worrying is the vast range of sources to which salmonellosis has been linked.

In addition to the clear risk to public health, *Salmonella* infections within fresh vegetable produce poses a financial and economic problem causing huge loses to the food-crop industry (Batz et al., 2012; Population Action International, 2012)

## 1.2 *Salmonella* and Crop Plant Ecology

The relationship between human pathogens and food has been studied comprehensively for some years. More recently the relationships and mechanisms between human pathogens, fresh vegetable produce and animal hosts/vectors has been explored. According to one report, there have been over 1900 illness's related to consumption of raw tomatoes between 1990 and 2010 (Bennett et al., 2015). When considering plants like tomatoes as vectors for human pathogens, there are number of points to note between initial germination of a seed and being eaten by consumer. These can be split broadly into pre-harvest and post-harvest. *Salmonella* may infect tomatoes at any stage of the production cycle although it is more common in the post-harvest phase (Devleesschauwer et al., 2017).

Crop contamination can occur in a number of ways, namely via the soil, water or potentially seeds and natural fertilisers (Barak and Schroeder, 2012), however the majority of these avenues originate from animal reservoirs, specifically faeces.

Water is essential for crop growing from use in irrigation to as a fertiliser diluent. Crops may easily be contaminated with pathogens if the water source used for irrigation is itself contaminated. Often the contaminated faeces can be moved from initial animal grazing sites via natural weathering and rain runoff. The contaminated faeces may often then reach reservoirs of surface water where the pathogens can persist (Cooley et al., 2007; Lewis et al., 2005; Luo et al., 2015). Surface water is often used to irrigate vegetable crops, with a reported 57% of vegetable farmers in the US doing so (Bihn et al., 2013). To further this issue many tomatoes growers now opt for drip hydroponic systems. The water source for these systems may vary, however some growers recollect field runoff to reapply to other crops, thus adding another avenue of potential contamination (Richard et al., 2006). In addition, it has been shown that irrigation regimes and water content of resulting fruit in themselves can have great effect on the ability of pathogens like *Salmonella* to proliferate in tomatoes. For example, it was reported in laboratory tests that *Salmonella* may proliferate at a 10-fold increase in water congested tomatoes (Marvasi et al., 2013).

It has also been reported that soil may play a part in the contamination of vegetable crops. Manure is a major source of fertiliser for many vegetable farmers (Elder et al., 2000). For the most part, it recycles essential nutrients into the soil needed for growing crops and increases microbial diversity at the same time. This however, is problematic if the faeces used in the manure is contaminated with human enteric pathogens. A survey found that of

29 cattle farms tested, 72% of them contained a faecal sample positive for *E. coli* OH157:H7 (Elder et al., 2000). The ability of a contaminated manure sample to effectively contaminate a crop is of course dependent on factors such as how long and what kind of conditions the manure may be stored in. However, *Salmonella* may persist in soil for some time, this can vary greatly depending on the composition of the soil as well as normal environmental pressures such as pH and temperature. In addition, the use of manures as fertiliser has been shown to increase the length of time that *Salmonella* may survive and persist in soils (Holley et al., 2006). Recent studies suggest that even dust particles carried via wind can contaminate tomato blossoms causing the resulting fruit and the calyx to be infected with *Salmonella* (Kumar et al., 2017).

Whilst *Salmonella* has the ability to infect the whole phyllosphere of the tomato plant, for the most part only the carpospohere is sold and consumed as product. This however, is of little consequence as *Salmonella* may persist as attachments on the fruiting body, the rhizophere or the entire phyllosphere. Results have shown that seeds sown in *Salmonella* contaminated soil have resulted in a the presence of *Salmonella* in the rhizosphere and resulting phyllosphere (Barak et al., 2008).

It is also important to note that these methods of infection may all be exacerbated if the phyllosphere and indeed the rhizosphere of the tomato plant has open wounds. Enteric bacteria such as *Salmonella* do not have the ability to penetrate the epidermis of the plant and so direct entry and infection may occur via open wounds in the plant, open stomata, root systems or flowering blossoms (Barak and Schroeder, 2012; Guo et al., 2001; Lopez-Velasco et al., 2012).

## 1.3 *Solanum lycopersicum* Anatomy

As previously mentioned, *Salmonella* may persist in tomatoes at any stage of the production process, be it pre-harvest or post-harvest. However, *Salmonella* may also persist in many different parts of the tomato plant. This study focussed on the carpospohere of the tomato plant . Whilst most studies (including this one) represent *Salmonella* proliferation in tomatoes as a relative increase per tomato, it is important to note the internal anatomy of the tomato carpospohere and the difference in the tissue types.

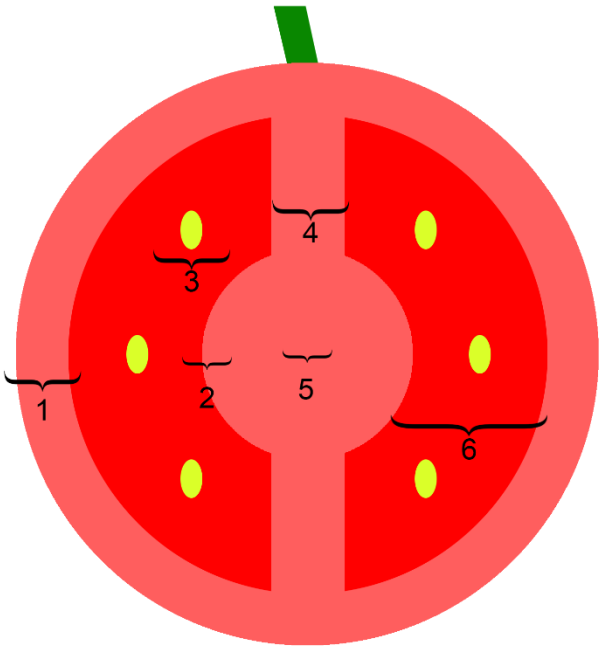


**Fig. 1 Diagrammatic Internal anatomy of tomato (longitudinal cross section).** 1-Pericarp 2-Placental tissue 3-Seed 4-Septa 5-Colmella 6-Locular Cavity.

Although it has been reported that *Salmonella* can grow in the seedlings, roots and leaves of the tomato plant, it is not currently known where exactly *Salmonella* may persist when inside the fruit of a tomato plant (Barak and Liang, 2008; Barak and Schroeder, 2012; Hintz et al., 2010). However as this study will demonstrate, *Salmonella* may be recovered from tomatoes when only the pericarp is inoculated. Tomatoes may be either bilocular or multilocular. Most varieties of tomato are multilocular apart from the smaller cherry varieties, including var. *cerasiforme* described in this study. These cavities contain the seeds of the plant and are filled with the fluid or juice of the fruit.

## 1.4 Plant Immune Responses

Plants immune systems employ a variety of different strategies to tackle both abiotic and biotic invaders. These are complex systems that allow the plant to recognise pathogen associated molecular patterns (PAMPs). In turn, the pathogens exhibit a host of strategies to allow them to persist within the plant and/or use as a host. Plant cells are able to recognise PAMPs via receptors on their cells called pattern recognition receptors (PRRs) (Río and Puppo, 2009). Depending on the PAMP recognised by the plant, it may begin to

transcribe particular defence proteins or release accumulated reactive oxygen species (ROS) into the intercellular space. The proteins produced by pathogens may also act directly to suppress the immunity of the plant (Río and Puppo, 2009). This results in a dynamic and complex set of interactions between plant and pathogen.

## 1.5 Reactive Oxygen Species In Plants - Hydrogen Peroxide and Tomatoes

One of the major and most reactive ROS found in plants is hydrogen peroxide ($H_2O_2$) and it has multiple functions. It has been reported that $H_2O_2$ produced by NADPH oxidases can regulate cell growth by controlling the activation of $Ca^{2+}$ channels in plants (Foreman et al., 2003). Not only this but $H_2O_2$ produced as a response to pathogen infection acts to stiffen, lignify and alter the composition of cell walls in plants to act as a barrier to invading pathogens (Cosgrove, 2005; Río and Puppo, 2009). Whilst the roles of $H_2O_2$ are plentiful in plants, the concern of this study is the role of $H_2O_2$ as a pathogen responses. Fruiting plants are no exception when $H_2O_2$ production is concerned, hydrogen peroxide has been found to be produced in apples, tomatoes and even detected in a variety of fruit juices (Lu et al., 2009; Nasirizadeh et al., 2016).

There is sufficient evidence to suggest plants like tomatoes exhibit microbursts of $H_2O_2$ when faced particular stressors, including invading pathogens (Río and Puppo, 2009). However, hydrogen peroxide may be produced as a by-product from photosynthesis as well as a secondary metabolite of reactions catalysed by enzymes such as amino acid oxidases and glucose oxidases (Asada, 1999). This means that even in the absence of a pathogen and/or immune response, plants may exhibit certain concentrations of hydrogen peroxide in the fruiting body. The particular concentration of hydrogen peroxide in plants may well vary between species and most importantly vary between varieties and cultivars, as this study will describe.

The genes which regulate hydrogen peroxide production vary in plants and some have been well described in tomatoes. For example, *RBOH1* codes for NADPH oxidase in *Solanum lycopersicum.* It has been shown that under stressors such as low temperature and drought, *RBHO1* is upregulated in tomato species (Mei et al., 2017). *RBOH1* is one such example of genes regulating $H_2O_2$ production in tomatoes, however, how this gene is regulated and the relative levels of expression in different varieties of tomatoes in not yet known.

## 1.6 Pathogen Oxidative Stress Response

Pathogens like *Salmonella* are far from defenceless even when faced with a multitude of plant defence mechanisms. Considering the number of ways *Salmonella* may enter and persist in a tomato plant, there are also a number of key metabolic and physiological characteristics that *Salmonella* has to ensure its proliferation. Attachment to plant surface can often be key to the colonisation of *Salmonella* in tomatoes. This is controlled by *agfD* which regulates the extracellular matrix of *Salmonella* (Barak et al., 2007). Identification of *yihT* gene in *S. enterica* sv. Tyhphimurium showed that this gene involved O-antigen capsule formation contributed to the proliferation success of *Salmonella* in tomatoes, *yihT* deletion mutants proliferated up to 3 logs less in green tomatoes (Marvasi et al., 2013).

*Salmonella* also has a number of ways of combatting hydrogen peroxide induced oxidative stress. These enzymes can broadly be split into two classes. There are the catalases that are able to degrease hydrogen peroxide into molecular water and oxygen. There is also the peroxiredoxin-type peroxidases (peroxiredoxins), which with the use of NADH or NADPH can reduce hydrogen peroxide to water (Horst et al., 2010).

The most well described catalases in *Salmonella* are KatE, KatG and KatN. KatE is controlled by the RpoS regulon whereas KatG is part of the OxyR regulon (Ibanez-Ruiz et al., 2000; Morgan et al., 1986). All of these contribute to the overall virulence and ability of *Salmonella* to proliferate. These catalases are an important first defence when faced with oxidative stress, as they require no reductant unlike the peroxiredoxins (Hebrard et al., 2009). Plant immune responses may well include microbursts of $H_2O_2$ and so producing reductants in such quick succession could prove problematic (Hebrard et al., 2009). However, studies have shown that single and double deletion mutants of KatG and KatE seemed to show no increased susceptibility to $H_2O_2$ producing macrophages in mice (Buchmeier et al., 1995). The role of these catalases in *Salmonella* proliferation within tomatoes has yet to be studied, and so their function and importance may well differ compared to when confronted with animal immune systems.

*Salmonella* also contains peroxiredoxin proteins AhpC and AhpF. These alkalyl hydrogen peroxide reductases are regulated by the OxyR regulon. Homologous genes of AhpC in *E. coli* were found to be more effective at scavenging $H_2O_2$ in trace amounts than the catalases such as KatE and Kat G, which respond at higher concentrations of $H_2O_2$. Thus it was suggested that peroxiredoxins form the primary oxidative stress response. In

addition, double deletion mutants of the Ahp and Kat genes in *E. coli* showed no ability to scavenge endogenous $H_2O_2$ (Seaver and Imlay, 2001).

## 1.7 Tomato CultiIvar Susceptibility

The ecology between *Salmonella* and tomato plants is complex and dynamic and both organisms have a varied set of mechanisms which allow them to defend themselves. As previously mentioned, tomatoes have been cited in a number of *Salmonella* outbreaks and the ability of *Salmonella* to persist in all parts of the tomato plant is clear. However, the ability of *Salmonella* to proliferate in tomatoes is not uniform across varieties and cultivars. This is seen in other host plants of enteric pathogens. The ability of *E. coli* O157:H7 to colonise lettuce plants was found to differ significantly across a variety of cultivars (Quilliam et al., 2012). There is also evidence to suggest that this is the case with *Salmonella* and a variety of tomato cultivars (Barak et al., 2011). A recent study screened 31 different cultivars of tomatoes, some known to be resistant to *Salmonella* and others of unknown susceptibility. The range in ability of *Salmonella* to proliferate in these cultivars varied greatly. In fact, there was found to be a range of 10 – 100 fold increase in *Salmonella* proliferation across the 31 varieties of tomato that were tested. However, of note was that the cherry varieties (such as var. *cerasiforme* described in this study) were generally more resistant to *Salmonella* than other larger cultivars such as Roma (Marvasi et al., 2014). This study however, chose not to explore this trend at the time.

There is good understanding of the mechanisms that govern plant-pathogen responses as well as the pathogen response itself. However, there is not yet a comprehensive understanding of what and how these mechanisms relate to the cultivar dependant ability of *Salmonella* proliferation.

The aim of this study is therefore to relate the cultivar dependent proliferation of *Salmonella* in *Solanum lycopersicum* var. *careasiforme* and var. *alicante* to the plant-pathogen response of $H_2O_2$ production. This will be done through a novel methodology of tomato intercellular fluid extraction and analysis.

# 2. METHODOLOGY

## 2.1 *Salmonella* Proliferation Test

### 2.1.1 *Salmonella enterica* (LT2) sv. Typhimurium Preparation

*Salmonella enterica* (LT2) sv. Typhimurium ATCC 14028 was obtained in stock from Middlesex University. This stock was then inoculated into 10ml of lysogeny broth (LB) and incubated for 24 hours at 37°C in 100rpm shaking incubator.

LB was washed from the inoculant using centrifugation. One ml of inoculant was placed in a 1.5ml Eppendorf tube and centrifuged at 8000rpm for 1 minute. The resulting supernatant was discarded and the pelleted cells kept. Cells were washed in this fashion three times over. The final pelleted cells were resuspended in 1ml of phosphate buffer saline (PBS).

Following this, 100µl of *Salmonella* suspension was pipetted into 900µl of PBS to make a $10^{-1}$ dilution. This was serially diluted until a *Salmonella* suspension of $10^{-5}$ was achieved. This dilution contains approximately $3x10^5$ cell/ml. *Salmonella* dilution $10^{-5}$ was used as inoculum for all tests in this study.

### 2.1.2 Tomato Preparation and Infection

Tomatoes of variety *cerasiforme* and *alicante* were bought on the morning of the experiment day. All tomatoes were washed thoroughly with deionised ($dH_2O$) prior to infection. For each proliferation test six tomatoes of var. *cerasiforme* were used and six tomatoes of var. *alicante* were used. The following methods were applied to all six biological replicates.

Using a sterile pipette tip, three shallow wounds were made in the pericarp of each tomato. This was done to ensure that the wound only punctured the pericarp and did not enter the locular cavities. Each wound was inoculated with 3µl of $10^{-5}$ *Salmonella.* All tomatoes were then placed into an empty plastic box that had previously been disinfected. The tomatoes were left to incubate at room temperature for 72 hours.

Following this,10µl of the initial $10^{-5}$ diluted inoculum was plated out on Xylose Lysine Deoxycholate (XLD) and incubated at 37°C for 24 hours. Colonies were counted after 24 hours and recorded as initial CFU inoculum count.

### 2.1.3 Tomato Harvest

After 72 hours of incubation, each tomato's weight was recorded and then placed in their own stomacher bag. If the tomato weighed 50g or less, 50ml of PBS was added to the stomacher bag. If the tomato weighed 50g or more, 100ml of PBS was added. Each tomato was then placed into a stomacher bag and stomached for 1 minute at 225rpm to homogenise the sample.

Following this, 20µl of each tomato sample was plated on XLD in duplicate. All plates were then incubated at 37°C for 24 hours.

Plates were then counted and recorded for black colonies of *Salmonella.* The increase of *Salmonella* (log) proliferation was calculated using the below formula

$$Log\left(\frac{Nfinal\ x\ Dilution\ factor\ x\ (vegetable\ weight + PBS)}{N\ initial}\right)$$

Where: $N_{initial}$ = Initial inoculum $10^{-5}$ *S.* Typhimurium on XLD counted as CFU

$N_{final}$ = Final CFU count on XLD

## 2.2 Quantification of Intercellular Hydrogen Peroxide

### 2.2.1 Intercellular Fluid Extraction

Using a sterile scalpel, 1cm x 1cm (approximately) sections of the tomato pericarp were cut. Tomato var. *cerasiforme* and var. *alicante* have varying pericarp thicknesses and so enough sections were cut so each sample equalled 1.5g ± 0.1g. The samples of tomato were cut carefully to ensure that as little of the locular cavity was taken as possible. The pericarp sections were submerged in 50mls of distilled water (or so all sections were submerged). Separate vessels are used for the cherry and Alicante cultivars respectively. The vessels are placed into separate vacuum pump bell jars and were subjected to a

vacuum for 15 mins (where vacuum pressure = 50kPa). The sections of pericarp were then removed and blotted gently with a paper towel to remove any excess water. The samples were then placed into 1.5ml Eppendorf tubes (approximately two pieces per tube). The samples were then centrifuged at 6000xg for 5 mins. The samples were then carefully removed from the Eppendorf tubes with sterile forceps and discarded. The remaining effluent was then transferred to a fresh Eppendorf tube and the sample' total yield was recorded in µl/g.

Samples were then subjected to deproteinisation using Abcam 10kD spin columns. To do so, 500µl of intercellular fluid was placed in the spin column and centrifuged at 11000xg for 3 mins. The effluent was then recollected and transferred to a fresh Eppendorf tube.

### 2.2.2 Hydrogen Peroxide Fluorometric Assay

The intercellular fluid extracted from each cultivar of tomato were then tested for the level of hydrogen peroxide using the Abcam Hydrogen Peroxide Assay Kit (Colorimetric/Fluorometric) (ab102500). Both deproteinised and non-deproteinised samples of both cultivars were tested.

Firstly, standards of hydrogen peroxide were set up. To do so, 10mM of hydrogen peroxide standard was made by diluting 10µl of 0.88M hydrogen peroxide into 870µl of $dH_2O$. Following this a 0.1mM standard was made by diluting 10µl of 10mM hydrogen peroxide into 990µl of $dH_2O$. Finally a 10µM standard was made by diluting 100µl of 0.1mM hydrogen peroxide into 900µl of $dH_20$. Six standards were made using this concentration ranging from 0nmol/well to 0.5nmol/well.

A clear bottom and topped 96 well plate was used for this assay. A reaction mix was then made for each well. This contained 48µl of assay buffer, 1µl of OxiRed Probe and 1µl of horseradish peroxidase (HRP). Following this, 1µl of either standard or sample was also added to its designated well. Both the standards and samples were measured in triplicate.

To ensure accurate results, the OxiRed Probe was added last to all wells to ensure all reactions started at a similar time.

The 96 well plate was then incubated at room temperature for 10 mins in a dark place. Following this the results were read in a 96 well plate-reader where Ex/Em = 535/587nm.

Readings from samples were compared against standard curve to find concentration of hydrogen peroxide in pmol.

## 2.3 Quantification of Intercellular Hydrogen Peroxide Post Infection Tomatoes

Tomato var. *ceriasiforme* and var. *alicante* were inoculated with $10^{-5}$ dilution of *Salmonella* as described in section 2.1.2. The tomatoes were incubated at room temperature for 72 hours.

Following incubation, sections of the pericarp where initial inoculation occurred, were cut and removed using a sterile scalpel. These sections were subjected to the intercellular fluid extraction method as described in section 2.2.1 The resulting samples were then quantified for hydrogen peroxide concentration as described in previous section.

## 2.4 *Salmonella* Proliferation Scavenger Test

Preparation of *Salmonella* inoculum was carried as described in section no 2.1.1 *Salmonella* proliferation tests were carried out as described in section 2.1. with the variations in this method being described below.

Six tomatoes of var. *cerasiforme* and six of var. *alicante* were each inoculated with 3x 3µl $10^{-5}$ *Salmonella* dilution and incubated at room temperature for 72 hours. After initial inoculation of the tomato, 3µl of 1XPBS was pipetted into the same wound from which *Salmonella* was first inoculated. This was done three times for each tomato. The tomato was treated with PBS in this fashion at 24 hours and 48 hours of incubation.

Six tomatoes of each variety were inoculated with 3x 3µl of $10^{-5}$ *Salmonella* dilution in the same fashion. After initial inoculation of the tomato, 3µl of 0.5mM sodium pyruvate was pipetted into the same wound from which *Salmonella* was first inoculated. This was done three times for each tomato. The tomato was treated with sodium pyruvate in this fashion at 24 hours and 48 hours of incubation.

Six tomatoes of each variety were inoculated with 3x 3µl of $10^{-5}$ *Salmonella* dilution in the same fashion. After initial inoculation of the tomato, 3µl of 0.5mM mannitol was pipetted into the same wound from which *Salmonella* was first inoculated. This was done three times for each tomato. The tomato was treated with mannitol in this fashion at 24 hours and 48 hours of incubation.

After the full 72 hours incubation, all tomatoes were weighed and recorded. They were then placed into stomacher bags and homogenised as described in section 2.1.3 Following this, 20µl of resulting homogenised samples were plated on XLD in duplicates and incubated at 37°C for 24 hours. Colonies were counted and recorded on all plates.

## 2.5 Hydrogen Peroxide Scavenger *Salmonella* Growth Curve

### 2.5.1 M9 Growth Medium Preparation

A stock solution of M9 was prepared to be used as growth medium in the *Salmonella* growth curves. 25ml of 2xM9 was added to 2ml of D-Glucose. Following this 0.2ml of 1M $MgSO_4$, 10µl of 1M $CaCl_2$, and 22.8ml of $dH_2O$ was added. This resulted in 50ml of stock M9. Where more M9 was needed, the formula was adjusted accordingly.

### 2.5.2 Preparation of Reaction Mix and Plate

Initially, stocks of each sample were made. This was done by pipetting 1ml of M9 stock into a 1.5ml Eppendorf tube. To this, 1µl of 0.5mM sodium pyruvate was added as well as 1µl of undiluted *Salmonella*. (*Salmonella* was prepared and washed as described in section 2.1.1 with the addition of 0.5mM sodium pyruvate added to washing solution. However, no serial dilutions were made for this test). This was done in triplicate to ensure there were three biological replicates.

This same process was repeated twice, substituting 0.5mM sodium pyruvate for 0.5mM mannitol and then again substituting for 1XPBS. A tabulated version of this can be seen in Table. 1

**Table. 1** Reaction mix for each scavenger treatment in *Salmonella* growth curve.

| | x 3 | x 3 | x 3 |
|---|---|---|---|
| COMPOSITE | SODIUM PYRUVATE TREATMENT | MANNITOL TREATMENT | PBS TREATMENT (CONTROL) |
| M9 | 1ml | 1ml | 1ml |
| 0.5MM SODIUM PYRUVATE | 1µl | n/a | n/a |
| 0.5MM MANNITOL | n/a | 1µl | n/a |
| 1X PBS | n/a | n/a | 1µl |
| *SALMONELLA* (UNDILUTED) | 1µl | 1µl | 1µl |

Following this, 200µl of each sample was pipetted into a clear bottomed 96 well plate. Each biological replicate from all treatments was plated so four technical replicates of each sample existed. A  blank containing 200µl of 1xPBS was also plated. An example of how this was plated can be seen in Fig. 2

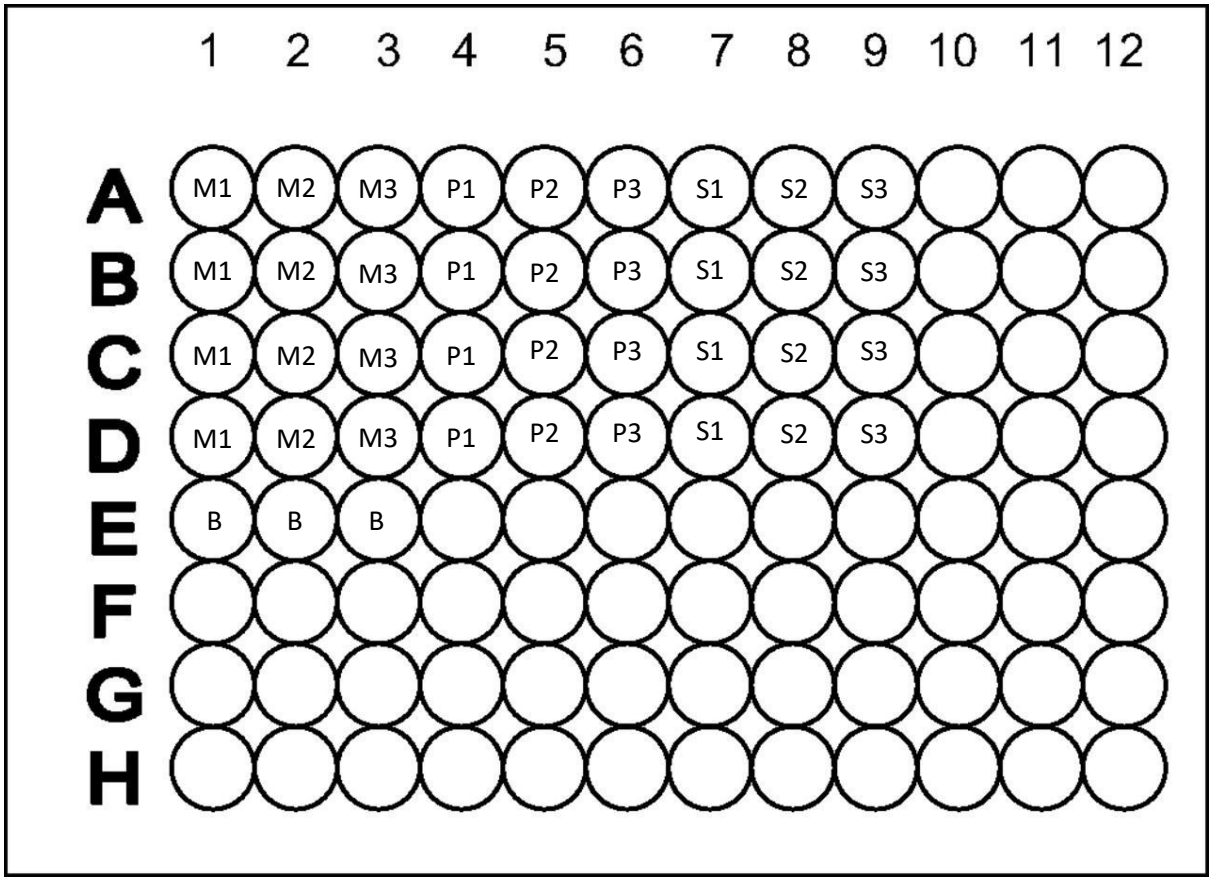


**Fig. 2** Example of 96 plate used for hydrogen peroxide scavenger growth curve. M1, M2 and M3 represent the biological replicates of the mannitol treatment. P1, P2 and P3 represent the biological PBS (control) treatments and S1, S2 and S3 represent the three sodium pyruvate treatments.

The plate was the incubated at 37°C for three hours before placing in the 96 well plate reader. The growth of *Salmonella* was measured every 30 mins for 14.5 hours. This was represented by absorbance values at 590nm.

## 2.6 RNA EXTRACTION

Three shallow wounds in the pericarp of var. *cerasiforme* and var. *alicante* tomatoes were made, spaced equally apart. Into each of these wounds 3µl of $10^{-5}$ *Salmonella* dilution was pipetted. This was done in triplicate for each cultivar of tomato. The tomatoes were

then left for 72 hours in a sterilised Perspex box, left at room temperature and out of direct sunlight.

2.6.1 Harvest and Sample Preparation

After the 72 hour incubation period the tomatoes were removed from the box for harvest of biological material. Using a sterile scalpel, sections of the pericarp surrounding the original wound made were cut and removed from the tomato, (after 72 hours, Salmonella will only proliferate in immediately surrounding areas). This was done for all three wounds on each of the six tomatoes (three var. *cerasiforme* and three var. *alicante*). Each removed section of pericarp was placed in its own sterile stomacher bag, along with 50ml of 1XPBS. Each sample was then placed into the stomacher for one minute at 225rpm. After all samples are fully homogenised, a sub sample of 1ml was taken from each and pipetted into a sterile 1.5ml micro centrifuge tube. Each sub sample was then spun at 100xg for 1 min, this ensured any large parts of biological material left from the homogenisation of the tomato are forced to the bottom of the tube. The liquid sample remaining in the tube was then transferred to a new sterile 1.5ml micro centrifuge tube, this was done for all samples. All samples were then spun at 11000xg for 3 mins to ensure cells were pelleted. The remaining effluent was discarded.

2.6.2 RNA Extraction

*All reagents used in this step were supplied by the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare)*

Firstly, the pelleted cells from each sample were resuspended in 100µl of TE buffer than contained 0.2mg/ml of lysozyme. Each sample was vortexed and the incubated at 37°C for 10 mins. Following this, 350µl of Lysis solution and 3.5µl of β-Mercaptoethanol was added and mixed by vortexing. The viscosity of the sample was reduced by filtering through a RNAspin Mini Filter. Following this, the RNAspin Mini Filter was placed inside a collection tube, and then the samples were loaded into the mini filter. One filter was used per sample. All samples were then centrifuged at 11000xg for 1 min. The filter was discarded and the collection tube containing the lysate was kept. For each sample, 350µl of 80% ethanol was loaded directly into the aforementioned collection tube.

For each sample a RNAspin Mini Column + collection tube complex was set up. Each sample lysate was then loaded directly into its own mini column. The samples were then centrifuged at 8000xg for 30 seconds. The mini column was then transferred to a new sterile collection tube. The previous collection tube was discarded. Following this 350µl of

desalting buffer was added to each mini column. All samples were then spun in a centrifuge at 11000xg for 1 min. For each sample, the flowthrough effluent was discarded but the collection tube kept and returned to the mini column.

The DNase reaction mixture was prepared according to the given protocol and in enough volume appropriate for this experiment. This was done by mixing 10µl of reconstituted DNase I with 90µl of DNase Reaction Buffer per reaction or sample. Following this, 90µl of this reaction mixture was loaded directly in the middle of the membrane at the bottom of the mini column. This was carried out for all samples and incubated at room temperature for 15 mins. For each sample, 200µl of wash buffer 1 was added to the mini column. They were then spun in a centrifuge for 1 min at 11000xg. Each mini column was then placed into a fresh collection tube and the old collection tubes were discarded. Each sample was then loaded with 600µl of wash buffer 2 and spun in a centrifuge for 1 min at 11000xg. The flowthrough effluent was discarded and the mini column was placed back in the collection tube. A third wash was carried out again with wash buffer 2 by adding 250µl to each sample and spinning in a centrifuge for 2 mins at 11000xg. The mini column was then placed into a nuclease free 1.5ml micro centrifuge tube, and all collection tubes from the previous step were discarded.

Finally, the RNA was eluted from the mini column. This was done by loading the mini column 100ul of RNase free water and then centrifuging at 11000xg for 1 min.

RNA quality and concentration was analysed using Nanodrop® Lite.

## 2.7 Statistical Analysis

All statistical analysis in this work was carried out using Minitab® v.17.2.1. Normality tests, tests for two variance were used to determine whether data were parametric or non-parametric. 2-sample t-tests were used to test for significance difference in parametric data and Mann-Whitney tests were used to test for significant difference in non-parametric data.

All statistical tests carried out in this work was done so with a significance of $\alpha = 0.05$, unless specifically stated.

# 3. RESULTS

## 3.1 *Salmonella* Proliferation Tests

*Solanum lycopersicum* var. *alicante* (Hessayon, 1997) and var. *cerasiforme* (Nesbit, 2002) were infected with *Salmonella enterica* serovar Typhimurium (14208). The increase in *S.* Typhimurium proliferation was measured in log (cfu/fruit). All proliferation tests were carried out according to methodology described in section 2.1

In this section, var. *cerasiforme* is denoted with its common name 'cherry' for ease.

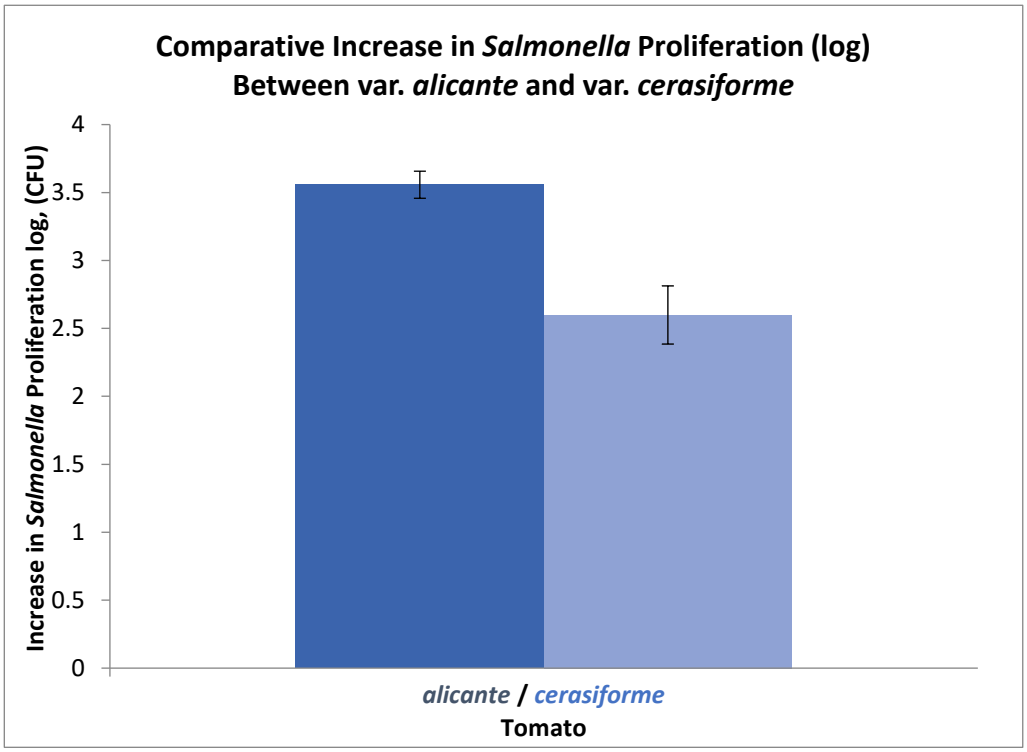


**Fig. 3** Increase in *S.* Typhimurium (14028) Proliferation (log) in *S. lycopersicum* var. *alicante* and var. *cerasiforme.* SE of var. *alicante* = 0.099623. SE of var. *cerasiforme* = 0.213772

Fig. 3 Shows the increase in *Salmonella* proliferation in var. *cerasiforme* and *alicante* of *Solanum lycopersicum.* Tomatoes were infected with $10^{-5}$ dilution of *S.* Typhimurium (14028) and incubated for three days as described in section 2.1. To ensure that the size, weight and overall growth area is accounted for in the difference between the two tomato varieties, growth is represented as a log increase in proliferation, this was done by using the formula described in section 2.1.3.

CFU count across biological replicas in both var. *cerasiforme* and *alicante* tomatoes initially indicates large variation. However tests for normality indicate that the average

CFU across biological replicates follow a normal distribution for cherry ($p$ = 0.301) and Alicante ($p$ = 0.077) (see appendix 1.6)

Further analysis of this data indicates that when comparing the average increase in *Salmonella* proliferation (CFU) both data (*cerasiforme* and *alicante*) have equal variances (Levene's $p$ = 0.098).

Each tomato variety's susceptibility to *Salmonella* proliferation is interpreted based on comparative increase in log. Analysis of the data in Fig. 3 shows the standard deviation of average CFU across all biological replicas in var. *alicante* and var. *cerasiforme* to be 111.4 and 54.9 respectively. However standard deviation of log increase in *Salmonella* proliferation in var. *alicante* and var. *cerasiforme* varieties was shown to be 0.267 and 0.574 respectively. In addition to this, the increase in *Salmonella* proliferation across all biological replicas showed normal distribution for both var. *alicante* ($p$ = 0.327) and var. *cerasiforme* ($p$ = 0.779) as well as equal variances (Levene's $p$ = 0.08).

Further analysis via 2-sample t-test shows that there is a significant difference in the increase in *Salmonella* proliferation between var. *alicante* and var. *cerasiforme* ($p$ = 0.004, Confidence Interval -1.534, -0.384). The above results and analysis indicates that var. *cerasiforme* has an inherent biological resistance to *S.* Typhimurium or potentially a higher amount of said resistance when compared with var. *alicante.*

## 3.2 Hydrogen Peroxide Quantification Analysis

As indicated in section 1.5 production of reaction oxygen species (like hydrogen peroxide) in plants is a primary form of defence against invading pathogens. It is possible that differences in the production amount of these reactive oxygen species between tomato varieties may help the explain the observed innate susceptibility and/or resistance of certain varieties for *S.* Typhimurium proliferation.

*Solanum lycopersicum* var. *alicante* and var. *cerasiforme* were analysed for concentration of hydrogen peroxide within their intercellular fluid respectively. The Fluorometric assay used standards of $H_2O_2$ ranging from 0 – 500pmol. Standard absorbance values were corrected using blank sample. Samples were checked against the standard curve.

In order to obtain the best and most representative data, sample preparations were optimised. All intercellular fluid was extracted using the method as described in section 2.2.1. After intercellular fluid extraction samples were deproteinised using Abcam® 10kD mini-spin columns and compared with samples that were not deproteinised. This

deproteinisation step was carried out to ensure that extraction of the intercellular fluid was not contaminated with proteins that may interfere with the sensitive Fluorometric quantification assay.

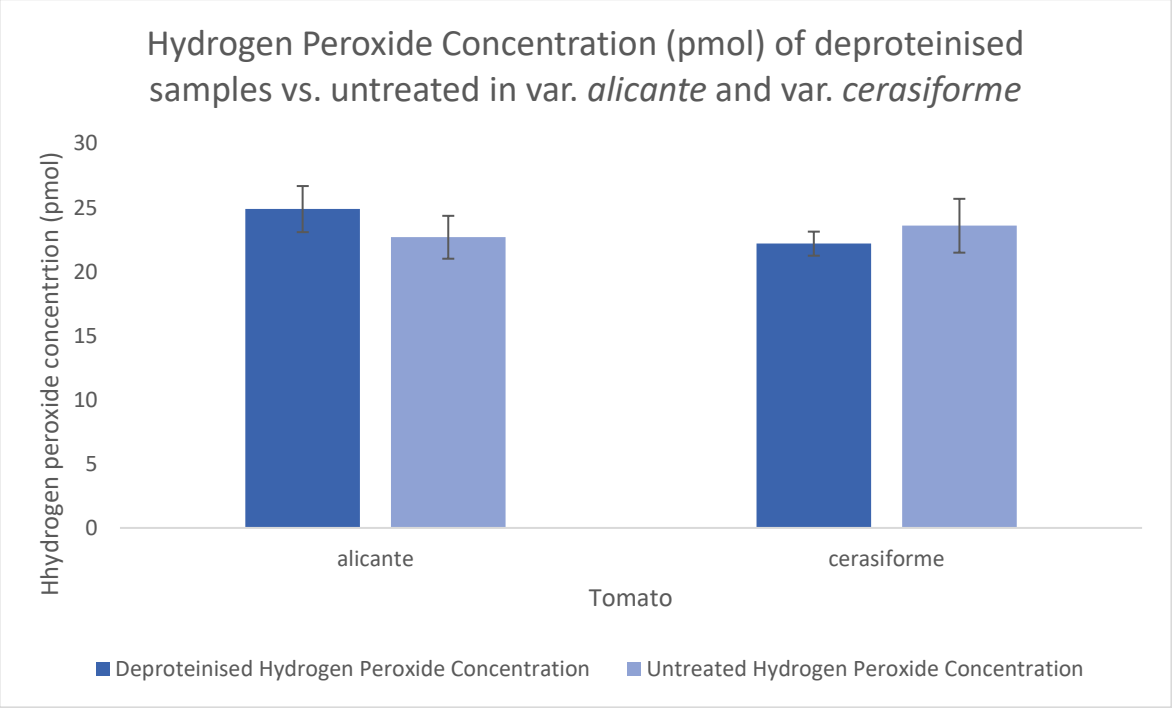


**Fig. 4** Hydrogen peroxide concentration (pmol) of deproteinised samples vs. untreated in var. *alicante* and var. *cerasiforme*. SE of var. *alicante* (deproteinised) = 1.8, SE of var. *alicante* (untreated) = 1.675, SE of var. *cerasiforme* (deproteinised) = 0.94, SE of var. *cerasiforme* (untreated) = 2.1.

The above figure shows the concentration of hydrogen peroxide in intercellular fluid samples taken from *S. lycopersicum* var. *alicante*. A 2-Sample t test indicated that there is no significant difference in hydrogen peroxide concentration between samples that were deproteinised and untreated samples. ($p$ = 0.17, 95% Confidence interval -1.27, 5.72). These results indicate that intercellular fluid extraction was successful.

Intercellular fluid samples from *S. lycopersicum* var. *cerasiforme* were also subjected to deproteinisation and non-deproteinisation. These samples were analysed and concentration of hydrogen peroxide in pmol was quantified. A 2-sample t-test indicates that there is no significant difference in concentration of hydrogen peroxide between samples that are deproteinised compared with those that are not ($p$ = 0.233, 95% confidence interval, -3.28, 11.36). It is important to note that the intercellular fluid used in the deproteinised and untreated samples came from a single tomato. This intended to remove any biological variation so the effect of deproteinisation could be properly observed.

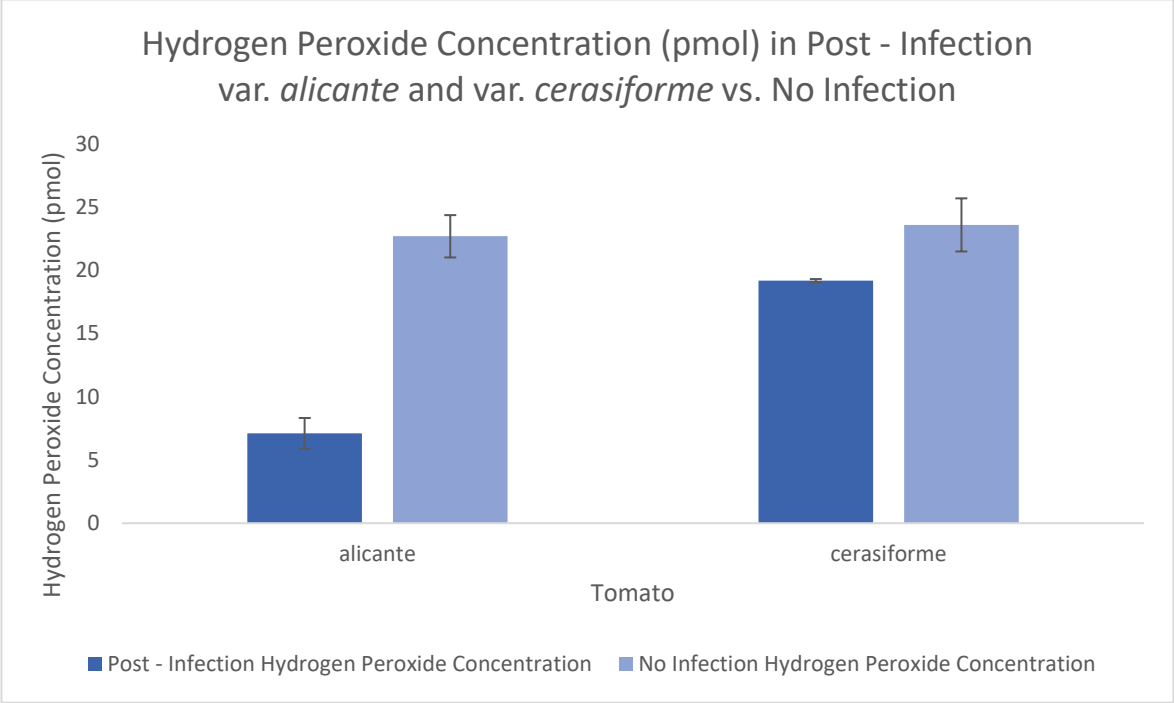


**Fig. 5** Intercellular concentration of $H_2O_2$ in *S. lycopersicum* var. *alicante* and var. *cerasiforme* post infection of *S.* Typhimurium vs. no infection. SE of var. *alicante* (post-infection) = 1.22, SE of var. *alicante* (no infection) = 1.675, SE of var. *cerasiforme* (post-infection) = 0.11, SE of var. *cerasiforme* (untreated) = 2.1.

The above figure shows the concentration of $H_2O_2$ in the intercellular fluid of *S. lycopersicum* var. *Alicante* and var. *cerasiforme* after infection of $10^{-5}$ *S.* Typhimurium and incubation for 1 day vs the intercellular concentration of hydrogen peroxide with no infection. Fluorescence readings from this test run yielded results that were close to or below the standard curve. However, statistical analysis shown a non-normal distribution ($p$ = <0.005) in the $H_2O_2$ concentration in var. *cerasiforme* post infection and a normal distribution for var. Alicante ($p$ = 0.287).

Further analysis via Mann-Whitney shows that there is a significant difference ($p$ = 0.0114 adjusted for ties, 95% Confidence Interval -4.62 -0.091) between the $H_2O_2$ concentration in var. *cerasiforme* post infection (Fig. 5) compared against untreated (no infection). The strength of this difference is however weak. Similarly, a 2-sample t-test shows a significant differences ($p$ = 0.001, 95% Confidence Interval 1.788, 5.331) between var. *alicante* post infection (Fig. 4) and untreated.

Analysis of these post infection results (Fig. 5) however are limited in reliability due to falling below standardised range for reference curve.

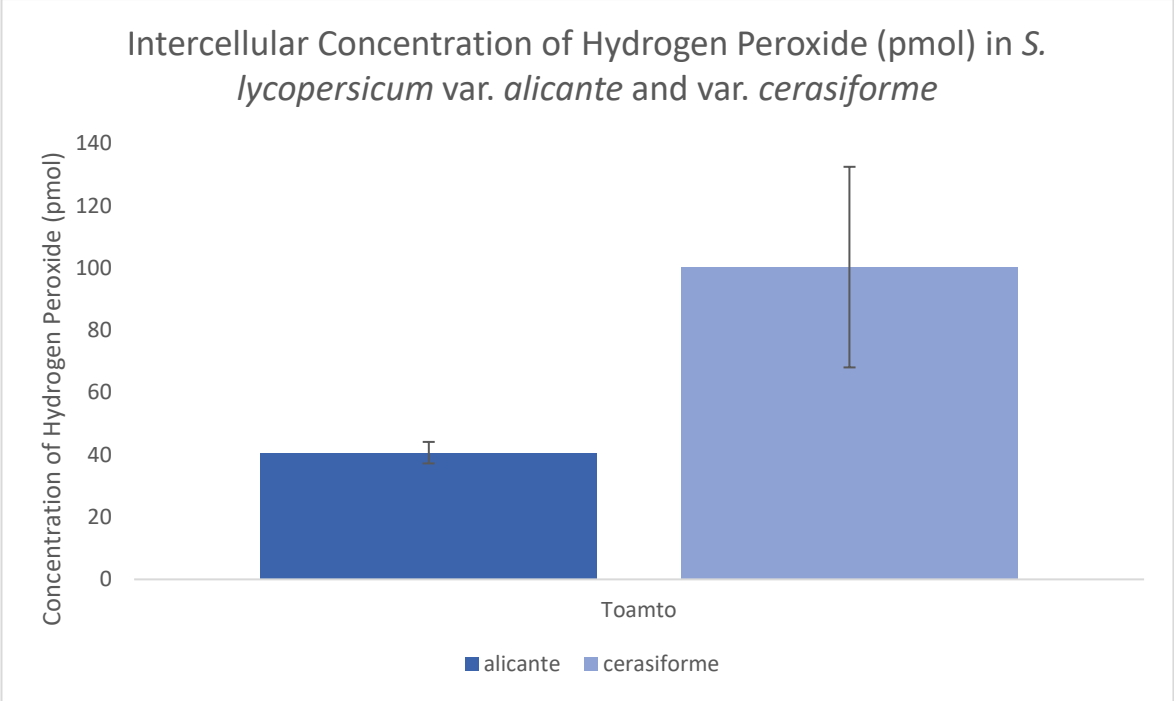


**Fig. 6** Intercellular concentration of $H_2O_2$ (pmol) in *S. lycopersicum* var. *alicante* and var. *cerasiforme.* SE of var. *alicante* = 3.4, SE of var. *cerasiforme* = 32.2.

The above figure shows the concentration in the intercellular fluid of *S. lycopersicum* var. *alicante* and var. *cerasiforme.* For this test, a new standard curve was run and concentrations were calculated against this. Var. *cerasiforme* was shown to have normal distribution ($p$ = 0.08) as was var. *alicante* ($p$ = 0.79). Further analysis via Mann-Whitney shows there to be a significant difference in the concentration of hydrogen peroxide between the two varieties of tomato ($p$ = 0.0304, 97% Confidence Interval 9.7, 162.7).

### 3.3 Proliferation Scavenger Test - Mannitol

With intercellular concentrations of hydrogen peroxide in both tomato varieties already established, it was important to confirm that the difference in *Salmonella* proliferation was due to the difference in hydrogen peroxide concentrations and no other variables. For this reason proliferation tests were carried out using hydrogen peroxide scavengers and was compared against controls. *Salmonella* proliferation tests were carried out on *S. lycopersicum* var. *alicante* and var. *cerasiforme* as dictated in section 2.1. Mannitol acted as a scavenger of $H_2O_2$

The data in Fig. 7 (full data available in appendix 1.1) shows the proliferation of *Salmonella* in var. *cerasiforme* and var. *alicante* when treated with mannitol vs. a control. Var. *alicante* (mannitol treatment), var. *alicante* (control) and var. *cerasiforme* (mannitol treatment) were shown to have normal distribution ($p = 0.55$, $p = 0.896$, $p = 0.835$) however var. *cerasiforme* (control) was shown to have non-normal distribution ($p = 0.017$). Further analysis via 2-sample t-test showed that there was no significant difference in the increase in proliferation in var. *alicante* tomatoes between the control and mannitol treatment ($p = 0.182$, Confidence interval -0.097, 0.445). However, analysis via Mann-Whitney showed there was a significant difference in the increase in proliferation in var. *cerasiforme* tomatoes between the control and mannitol treatment ($p = 0.013$, Confidence interval -0.842, -0.096).

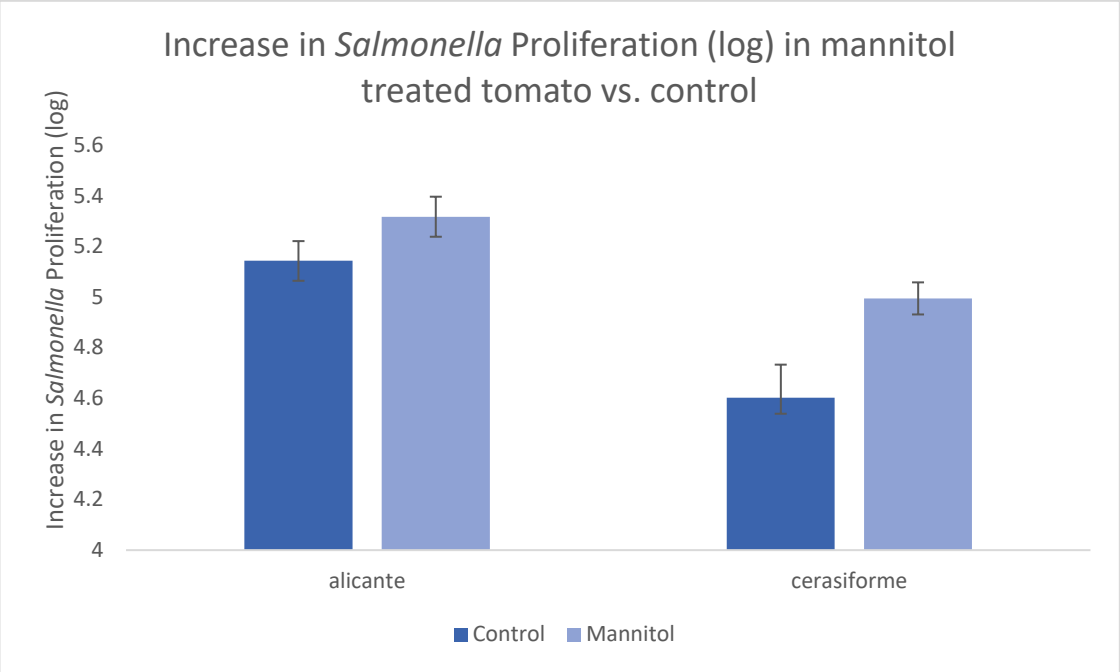


**Fig. 7** Increase in *Salmonella* proliferation (log) in *S. lycopersicum* var. *alicante* and var. *cerasiforme* when treated with Mannitol vs. control. Standard error of Cherry (control) shown as 0.130, standard error of cherry (mannitol) shown as 0.063, standard error of Alicante (control) shown as 0.077 and standard error of Alicante (mannitol) shown as 0.0791303

Further analysis via Mann-Whitney shows that there lies a significant difference between proliferation in var. *cerasiforme* and var. *alicante* in the control tomatoes ($p$ = 0.0082, Confidence interval -0.94, -0.195). These results are in keeping with and compliment the analysis of data from Table 2. And Fig. 3. However, 2-sample t-test analysis shows there also to be a significance difference between proliferation in var. *cerasiforme* and var. *alicante* varieties in the Mannitol treatment ($p$ = 0.016*,* Confidence interval, -0.570, -0.075).

Analysis of the data shown in Fig. 7 was carried out identify if significance difference lies between the estimated difference in the two treatments of both tomatoes. Estimates for difference from summarised t-tests were used as sample means and pooled standard deviations from original t-test used as standard deviation (shown in Table. 2).

**Table. 2** Summarised statistical data used to test significant difference in estimated difference in var. *cerasiforme* and var. *alicante* proliferation (control) and var. *cerasiforme* and var. *alicante* proliferation (mannitol)

| | Test | |
|---|---|---|
| | Summarised t-test of Cherry proliferation (control) and Alicante proliferation (control) | Summarised t-test of Cherry proliferation (mannitol) and Alicante proliferation (mannitol) |
| Estimate for difference | -0.323 | -0.541 |
| Pooled StDev | 0.1756 | 0.1756 |

Summarised t-test between showed there was no significance difference ($p$ = 0.057, Confidence interval -0.008, 0.444) between the estimated differences of tests described in Table. 2 with an estimate for difference at 0.288.

### 3.4 Proliferation Scavenger Test – Sodium Pyruvate

*Salmonella* proliferation tests were carried out on *S. lycopersicum* var. *alicante* and var. *cerasiforme* as dictated in section 2.4. Sodium Pyruvate acted as a scavenger of $H_2O_2$

The data in Fig. 8 shows the proliferation of *Salmonella* in var. *cerasiforme* and var. *alicante* when treated with sodium pyruvate vs. a control. Var. *alicante* (sodium pyruvate treatment), var. *alicante* (control) and var. *cerasiforme* (sodium pyruvate treatment) and var. *cerasiforme* (control) were shown to have normal distribution ($p$ = 0.284, $p$ = 0.866, $p$ = 0.633, $p$ = 0.532 respectively). Further analysis via 2 samples t-test showed that there was no significant difference in the increase in proliferation in var. *alicante* between the control and sodium pyruvate treatment ($p$ = 0.720, Confidence interval -0.290, 0.208). Similarly, analysis via 2-sample t-test showed there was no significant difference increase in proliferation in var. *cerasiforme* between the control and sodium pyruvate treatment ($p$ = 0.193, Confidence interval -0.0649, -0.282).

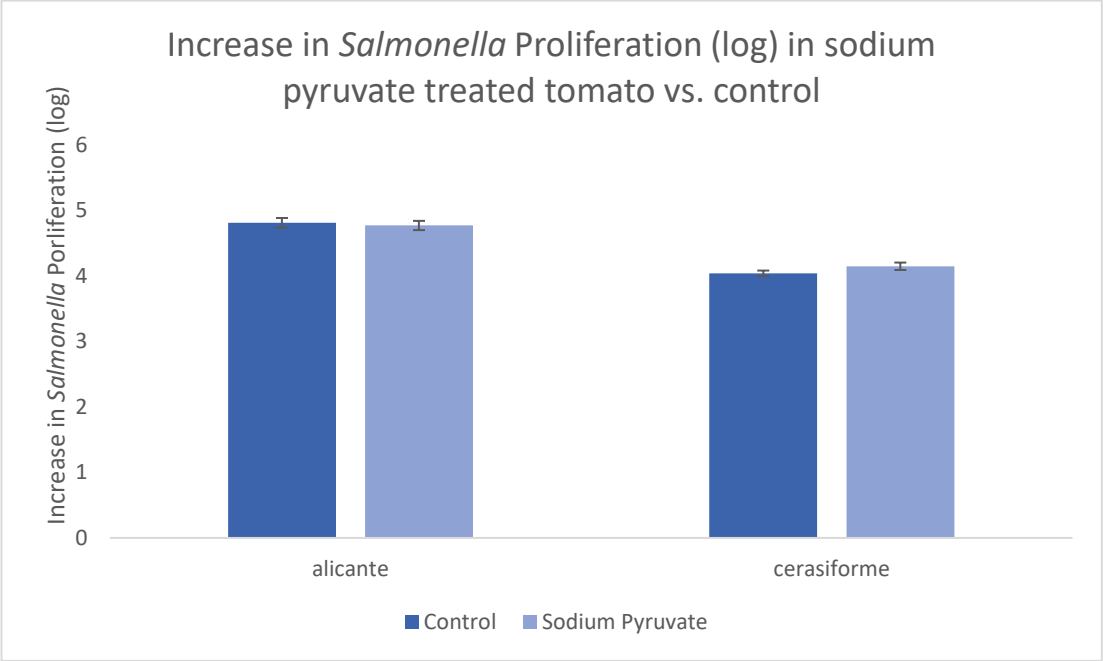


**Fig. 8** Increase in *Salmonella* proliferation (log) in *S. lycopersicum* var. *alicante* and var. *cerasiforme* when treated with sodium pyruvate vs. control. Standard error of Cherry (control) shown as 0.105, standard error of cherry (sodium pyruvate) shown as 0.138, standard error of Alicante (control) shown as 0.073 and standard error of *alicante* (sodium pyruvate) shown as 0.071.

Analysis via 2-sample t-test shows that there lies a significant difference in the increase in proliferation between var. *cerasiforme* and var. *alicante* (control) ($p$ = <0.000, Confidence Interval 0.5646, 0.981). This analysis shares the same outcome with the data described in

Fig. 3 Fig. 7 and Fig. 8. Furthermore, there also lies a significant difference between the increase in proliferation between var. *cerasiforme* and var. *alicante* (sodium pyruvate treatment) ($p$ <0.000, Confidence Interval -0.843, -0.402). The estimate for difference is smaller however between the treated tomatoes (0.6299) and the control tomatoes (0.7726).

Further analysis was carried out to test for significance between the estimated differences in var. *cerasiforme* and var. *alicante* (control) and var. *cerasiforme* and var. *alicante* (sodium pyruvate). Estimates for difference from summarised t-tests were used as sample means and pooled standard deviations from original t-tests used as standard deviation (shown in Table. 3).

**Table. 3** Summarised statistical data used to test significant difference in estimated difference in var. *cerasiforme* and var. *alicante* proliferation (control) and var. *cerasiforme* and var. *alicante* proliferation (sodium pyruvate)

| | **Test** | |
|---|---|---|
| | Summarised t-test of Cherry proliferation (control) and Alicante proliferation (control) | Summarised t-test of Cherry proliferation (Sodium Pyruvate) and Alicante proliferation (Sodium Pyruvate) |
| Estimate for difference | -0.7726 | -0.6299 |
| Pooled StDev | 0.1617 | 0.1717 |

Summarised t-test showed there was no significance difference ($p$ = 0.169, Confidence interval -0.0718, 0.3572) between the estimated differences of proliferation in sodium pyruvate treated tomatoes (var. *cerasiforme* and *alicante*) and sodium pyruvate treated tomatoes (var. *cerasiforme* and *alicante*) with an estimate for difference at -0.1497.

### 3.5 Hydrogen Peroxide Scavenger *Salmonella* Growth Curve

It was important to ensure that results observed in scavenger proliferation tests were accurate and not confounded by other variables. To ensure that mannitol and sodium pyruvate did not positively contribute to the overall growth of *S.* Typhimurium, a number of growth curves were carried out.

Growth curves of *Salmonella* were carried out according to the protocol dictated in section 2.5. Three biological and four technical replicas were measures for growth over a 14.5 hour period. Three treatments were used; mannitol, sodium pyruvate and control (PBS).

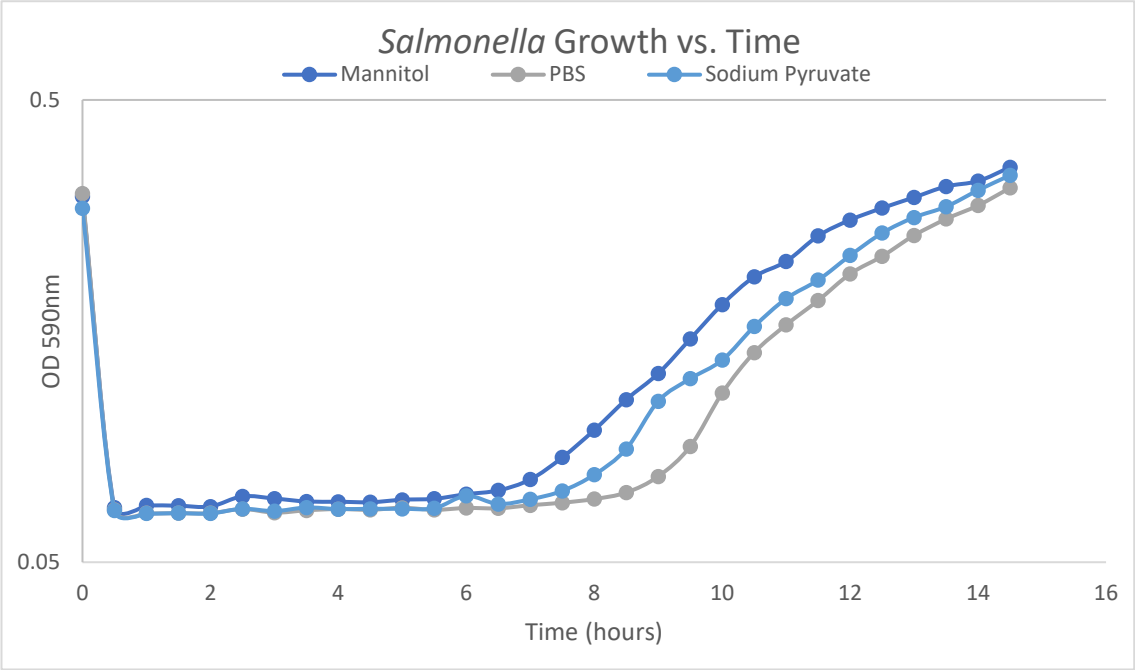


**Fig. 9**. Growth of *S.* Typhimurium (as indicated by OD: 590nm) over time when treated with mannitol, sodium pyruvate and control (PBS).

Clear and distinct phases of microbial growth are seen in Fig. 9, the lag phase and the exponential. Outliers are present at or near to time 0 and are not included in statistical analysis. Smooth curves have been drawn to represent a more exact record of growth over time. The stationary phase and death phase cannot be seen in Fig. 9. However, for the purpose of these tests, the exponential phase (logarithmic phase) is of primary interest. The growth curve shown in Fig. 9 also shows higher than normal OD values for all three treatments at Time 0. This is likely due to reading error and does not accurately depict growth over the first 30 minutes.

In order to compare the growth of *Salmonella* over time between the three treatments, the exponential phase of each growth curve was isolated and then linearised.

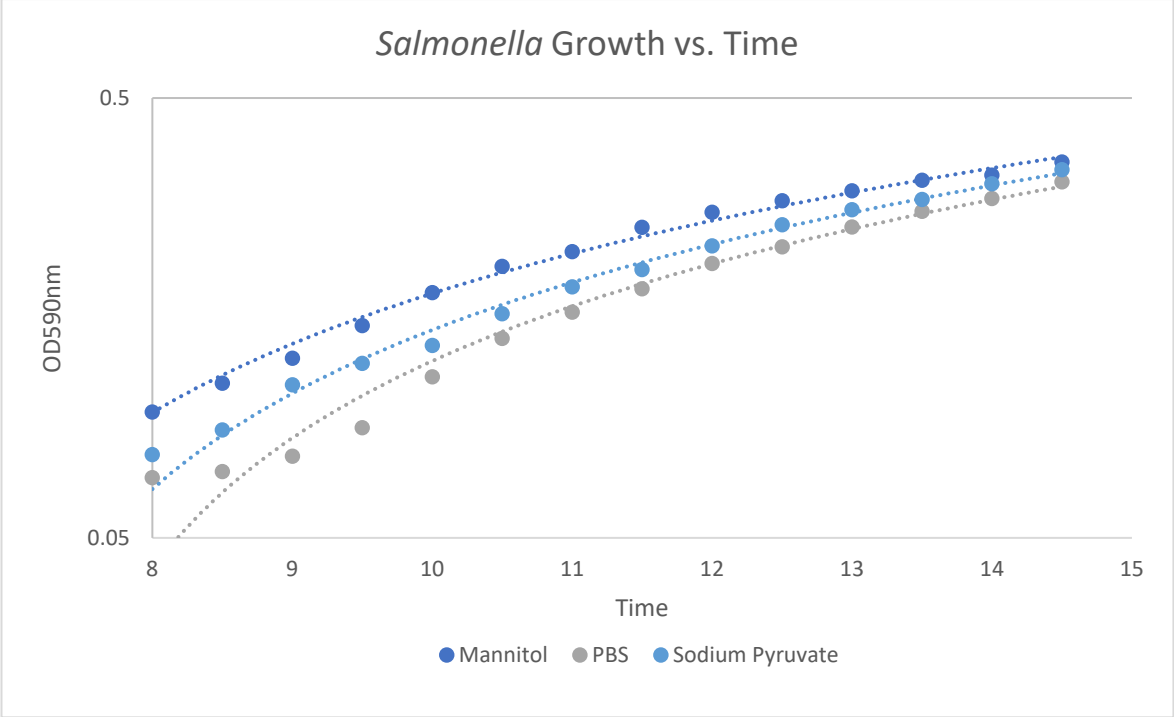


**Fig. 10** Linearised exponential phase of *Salmonella* growth when treated with Mannitol, Sodium pyruvate and control (PBS)

**Table. 4** Summarised Slopes and $r^2$ values for linear trend lines of growth (treatments and control)

| | Treatment | | |
|---|---|---|---|
| | **Control (PBS)** | **Mannitol** | **Sodium Pyruvate** |
| Slope | y= -0.0345x -0.1734 | y= -0.0381x -0.2052 | y= -0.0327x -0.1791 |
| $r^2$ | 0.9924 | 0.9933 | 0.9841 |

In order to test for significant difference between the linearised exponential phases of each treatment, linear regression was carried out. Linear regression allowed generation of coefficients and the standard error of these coefficients. Mannitol treatment was found to have coefficient of 0.0395 ($p$ <0.000), sodium pyruvate was found to have a coefficient of 0.0351 ($p$ <0.000) and control (PBS) was found to have a coefficient of 0.0353 ($p$ <0.000). Standard deviation was calculated by 'Standard Error of Coefficient x √n=12'. Coefficient of the linear regression was used as sample mean for a summarised 2-sample t-test.

Results of the 2-sample t-test showed that there lies a significant difference between the growth of *Salmonella* when treated with Mannitol compared against a control ($p$ = 0.003, Confidence Interval 0.00158, 0.0066). The estimate for this difference was found to be 0.00413.

Further analysis via 2-sample t-test showed that there is no significant difference between the growth of *Salmonella* when treated with sodium pyruvate compared against a control

($p$ = 0.884, Confidence Interval, -0.00267, 0.00231). The estimate for this difference was found to be -0.00018.

Whilst these tests reveal significant and no significant differences between treatments and control, in both cases the estimate for those differences is negligible. The sensitivity in these tests in determining the significance can be explained by the extreme linear relationship. Table. 4 shows the $r^2$ value from each linear trend line, all of which show an almost exact relationship with the relevant data set. Additionally, the slopes of each of these trend lines as described in Table. 4 are very similar. Therefore, any deviation (albeit negligible) may be statistically significant.

### 3.6 RNA Extraction and Quantification

The initial aim of this study included the analysis of specific genes, KatG and KatE that are involved in *Salmonella* immune responses. RT-PCR was then to be carried out to compare the relative expression of these two genes after inoculation within the two varieties of tomatoes (var. *alicante* and var. *cerasiforme*). The first step of this process involved RNA extraction, results of which can be seen below. However, time constraints and low concentrations of RNA did not allow for continuation of this method.

RNA was extracted from *Salmonella* after 72 hour incubation var. *cerasiforme* and var. *alicante* using the illustra RNAspin Mini RNA Isolation Kit.

**Table. 5** RNA quantification of *S.* Typhimurium after 72 hour incubation in var. *alicante* and var. *cerasiforme* including purity ratios

| | Biological Replicas | RNA Concentration (ng/ µL) | A260(Abs) | A280(abs) | 260/280 | 260/230 |
|---|---|---|---|---|---|---|
| Alicante | 1 | 5.6 | 0.141 | 0.059 | 2.39 | 0.47 |
| | 2 | 3.6 | 0.089 | 0.038 | 2.34 | 0.1 |
| | 3 | 5 | 0.126 | 0.058 | 2.18 | 0.55 |
| Cherry | 1 | 2.7 | 0.068 | 0.035 | 1.96 | 0.09 |
| | 2 | 4.2 | 0.105 | 0.051 | 2.08 | 0.62 |
| | 3 | 3 | 0.074 | 0.036 | 2.04 | 0.07 |

RNA extraction as shown in Table. 5 was found to have low yields, with an average yield of 4.7ng/µl for var. Alicante and an average of 3.3ng/µl for var. *cerasiforme*. In addition absorbance ratios of A260/A230 indicate that most extractions were considered impure with an average A260/A230 ratio of 0.37 for var. Alicante and an average of 0.26 for var. cherry. Samples considered pure normally show a A1260/A230 ratio of 2.0-2.2 A second indication of purity is represented by A260/A280 ratio, pure samples are considered to have a A260/A280 ratio of 1.8-2.0 (NanoDrop Technologies™, 2007). Samples extracted from var. cherry indicate on average an above normal ratio (2.02). Biological replicate 1 shows a 'pure' sample ratio of 1.96, however the yield is low at 2.7ng/µl.

However, subsequent gel electrophoresis of these samples showed entirely negative (absent) results, bar the reference ladder.

# 4. DISCUSSION

## 4.1 *Salmonella* Proliferation

The rationale for this study as explained in section 1.7 is based in part from Marvasi et al. (2014). This study found in a secondary aim that there existed a significant difference in the ability of *Salmonella* to proliferate in different varieties of tomato. This study tested 31 different varieties, but found most interestingly that the largest and most significant difference in *Salmonella* proliferation was between tomatoes of the cherry variety (of which there are multiple) and large tomatoes. For this study, two specific varieties were chosen to test; a cherry variety described as *cerasiforme* (Nesbitt and Tanksley, 2002) and a large variety describes as *alicante* (Hessayon and Hessayon, 1997).

Base line data had to be obtained in order to confirm findings from Marvasi et al. (2014). These results can be seen in Fig. 3. Analysis of these results found that there was a significant difference between the proliferation of *Salmonella* in var. *cerasiforme* and var. *alicante* ($p = 0.004$). With *Salmonella* proliferating up to 3.5 logs on average in var. *alicante* and 2.5 logs in var. *cerasiforme.* These results show the same trends seen in Marvasi et al. (2014). It is important to note that the varieties of tomatoes used in this study are not represented in the aforementioned paper. However, this further points to the assertion that cherry variety tomatoes do indeed show an increased resistance to *Salmonella* proliferation than varieties of larger size. If this inherent resistance to *Salmonella* proliferation is due to particular physical properties of the tomato, then it would follow that similar cherry varieties such as Large Red Cherry (Marvasi et al., 2014) and var. *cerasiforme* would share such properties.

It should be stated that the results presented in Marvasi et al. (2014) and this study are directly comparable in both methodology for proliferation tests as well as subsequent results. Both results are presented as an increase in *Salmonella* proliferation rather than a total number. The reason for this is that total growth of *Salmonella* does not accurately represent the ability of *Salmonella* to proliferate in the tomato. This increase is compared against a CFU count from the original inoculum (described in section 3.1). The equation used to calculate said increase in proliferation also accounts for the wet weight of the tomato which is imperative when comparing two varieties of tomato with varying sizes and weights.

## 4.2 Hydrogen Peroxide Quantification and Intercellular Fluid Extraction

Through data analysed in Fig. 3 as well as the data presented in Marvasi et al. (2014) it was clear there was an inherent biological disposition for tomatoes of the smaller cherry variety to possess a higher resistance to *Salmonella* proliferation than varieties of larger size such as var. *alicante* described in this study. The reason for these observed differences have yet to be described. It is likely however that the complexities of plant immune systems are responsible.

One of the common plant defence mechanisms is the production of reactive oxygen species (ROS), of which hydrogen peroxide is most common (Foreman et al., 2003). Hydrogen peroxide has multiple functions in plants such as regulation of cell growth and cell wall lignification (Cosgrove, 2005). However ROS such hydrogen peroxide are also an important part of the plant immune response. The rationale of this study therefore followed that the inherent difference in susceptibility to *Salmonella* proliferation between tomato varieties could be due to differences in efficacy of plant immune responses, specifically the concentration and/or production of hydrogen peroxide. In order to test this hydrogen peroxide was quantified in tomato var. *cerasiforme* and var. *alicante.*

*Salmonella* may persist in all parts of phyllosphere and rhizophere, with there being reports of growth on seedlings and roots (Barak and Schroeder, 2012; Guo et al., 2001). Due to *Salmonella* ability to produce complex extracellular matrices, it can also form attachments to the surface of the fruiting body and even the flowering plant (Lopez-Velasco et al., 2012). There is currently no evidence to suggest exactly where *Salmonella* may persist inside of the carpospohere. However, it is likely that *Salmonella* would normally persist within the pericarp of the fruiting body, most likely within the intercellular space, especially if its method of entry is via wounds in said pericarp.

Additionally there is evidence to suggest that plants recognise pathogen associated molecular patterns (PAMPs) which can cause the transcription and subsequent release of defence proteins or ROS like hydrogen peroxide into the intercellular space (Río and Puppo, 2009). If these PAMPs are recognised by the cells in the pericarp it stands to reason this is where pathogens may persist. Furthermore, it has been shown that *Pseudomonas solanacearum* (a common wilting pathogen in tomato and tobacco plants) will persist in the intercellular space in tobacco plants (Rathmell and Sequeira, 1975).

### 4.2.1 Intercellular Fluid Extraction

Hydrogen peroxide was quantified using samples of the pericarp rather than the whole fruiting body including locular cavities. This way an accurate representation of the cellular environment could be analysed. In addition to this, the methodology for measuring *Salmonella* proliferation in tomatoes involves inoculating the pericarp and so it was important to also measure this part of the carprophere for hydrogen peroxide. There exists within the literature, methodologies and protocols for extracting intercellular fluid from plants such as the methods described in Rathmell and Sequeira (1975). However, due to vast variation in morphology of plants and their fruiting bodies, there exists no standard protocol, and each example in the literature varies due to purpose of the study and the plant/fruit involved. In addition there exists no specific examples within the literature for extraction of intercellular fluid in tomato fruits whatsoever and so the methodology described in section 2.2.1 is novel. The methodology described in section 2.2.1 is based in part on vacuum infiltration, a method of inducing gene expression in plants via *Agrobacterium tumefaciens.*

### 4.2.2 Hydrogen Peroxide Quantification

Fig. 4 shows the concentrations of hydrogen peroxide in var. *alicante* and var. *cerasiforme* respectively. These initial tests also included samples that were deproteinised using 10kD spin columns. Samples were deproteinised in this way to ensure that the resulting samples from the method of intercellular fluid extraction contained as a little protein content as possible. This ensured that the fluorometric assay that quantifies hydrogen peroxide was not affected by large amounts of protein in the samples. As previously stated, plant cells may release proteins into the intercellular space (Río and Puppo, 2009). For example, taste modifying proteins are reported to be released into the intercellular space in *Richadella dulcifica* (Hirai et al., 2010). However the protein content within plant cells (specifically in cell walls) is much higher than in the intercellular space. Thus, if the novel methodology of intercellular fluid extraction works as intended, then there should be little difference in samples that are deproteinised compared to those untreated. The samples labelled deproteinised and untreated in Fig. 4 came from the same tomato (var. *alicante*) and same intercellular fluid. This was the same for var. *cerasiforme*.

Analysis showed that there was no significant difference in the hydrogen peroxide concentration when the same sample was deproteinised vs. untreated. This trend was seen for var. *alicante* and var. *cerasiforme* (Fig. 4). These results indicate that method of

intercellular fluid extraction does indeed extract majority intercellular fluid with little protein content, and not cell wall or cytoplasmic fluid.

## 4.3 Post Infection Hydrogen Peroxide Quantification

Hydrogen peroxide and other ROS are produced in plants as a by-product of photosynthesis and cellular respiration (Chakraborty et al., 2016). They also have multiple roles in controlling plant hormones like auxin (Río and Puppo, 2009). Because of the abundance of roles ROS like hydrogen peroxide play, its presence in plants both intracellularly and intercellularly is plenty. This means there is likely ROS like hydrogen peroxide present (albeit in unknown concentrations) in plants and their fruiting bodies without necessarily being produced via a stress response. Because of this, the literature describes plant defence systems as being pre-existing or activated defences. Pre-existing defences are those that are present with or without microbial infection and activated defences are those that are produced/triggered only in response to microbial attack and/or abiotic stress such as wounding (He et al., 2007; Jones and Dangl, 2006; Nurnberger et al., 2004).

It was therefore necessary determine if the level of hydrogen peroxide in var. *alicante* and var. *cerasiforme* changed after inoculation with *Salmonella*. Proliferation tests and hydrogen peroxide quantification were carried out as described in sections 2.1 and 2.2 respectively, the results for which can be seen in Fig. 4. Analysis showed that there lies a significant difference ($p = 0.001$) in the concentration of hydrogen peroxide between post infection and no infection in var. *alicante,* with the concentration of hydrogen peroxide actually decreasing in the post infection measured tomatoes. The trend is also observed for var. *cerasiforme* where a significant difference ($p = 0.0114$) was found between hydrogen peroxide concentration post infection vs no infection. In both cases however, concentration of hydrogen peroxide seemed to decrease in the post infection tomatoes.

The trend observed was an unexpected outcome. It would follow that in the presence of microbial attack, activated plant defences and immune responses would trigger further action in the tomato fruit. Plants plasma membranes contain pattern-recognition receptors (PRRs) which can recognise PAMPs and respond accordingly. An example is the cell surface receptor FLS2 in *Arabidopsis spp.*. This receptor can recognise the specific flagella protein flg22, which is present in multiple bacterial species (Gómez-Gómez and Boller, 2000). The recognition of flg22 by FLS2 elicits multiple defence responses such as

cell wall lignification but also micro-bursts of hydrogen peroxide. Flg22 is also a potent elicitor of defence responses in varying plant species, not just *Arabidopsis* (Felix et al., 1999; Gómez-Gómez and Boller, 2000; Zipfel et al., 2004). In addition to this, there have been many reported '*R'* (plant resistant proteins) genes in tomatoes that are produced as a response to microbial attack. Often these responses are specific to a particular microbial species or genus (Hammond-Kosack and Parker, 2003). In some cases these genes can code for resistance such as the *Ve* gene in *Lycopersicon esculentum* that gives resistance to *Verticillium dahliae* (Kawchuk et al., 2001).

It may be fair to assume therefore that the level of hydrogen peroxide in the intercellular space of tomatoes would be higher post infection of *Salmonella* compared to not. It is possible that the reason this trend is not observed in the results is due to methodological issues. As described in section 2.2.2, the fluorometric assay that quantifies hydrogen peroxide in biological samples, does so by comparing against a standard curve of known concentrations. When measured samples fall below this standard curve, accurately converting absorbance values to concentrations becomes difficult. Whilst Fluorometric assays are generally considered a sensitive means of detection, it is clear in this instance the level of hydrogen peroxide in the post infection tomato samples was too low to be accurately measured.

## 4.4 Tomato Ripeness and Post–Harvest Measurement of Hydrogen Peroxlde

### 4.4.1 Tomato Ripeness

It is important to note that both the proliferation tests and hydrogen peroxide quantification carried out var. *alicante* and var. *cerasiforme* were done so on the fruiting body post-harvest. Tomato fruits post-harvest are still 'biologically active' parts of the plant as a whole. As such they are subject to ripening from ethylene production and other similar biological activities (Zhong et al., 2008). However, the normal immune responses (activated or pre-existing) in the tomato fruit are compromised when they are post-harvest (Shi et al., 2007). In addition, it has been reported that there are great differences in different *Salmonella* serovars and their ability to persist in tomatoes pre and post-harvest. Shi et al., (2007) reported on testing five different serovars of *Salmonella* Typhimurium, that all were able to proliferate to high numbers in post-harvest ripe tomatoes. However, these same serovars had differing success when inoculated on the surface or in the tissue of pre-harvest tomatoes.

It must be stated that all tomatoes used in this study were purchased and not grown in controlled laboratory conditions. Whilst the particular variety of tomato was able to remain consistent for all experiments in this study, the stage of ripeness was not. The stage of ripeness of the tomato at point of inoculation can have great effects on the susceptibility of the tomato to *Salmonella* proliferation. The ability of *Salmonella* to be able to proliferate and persist in tomatoes increases with ripeness. It has been reported that in 31 different tomato varieties tested, *Salmonella* proliferation was consistently increased in ripe versions of these tomatoes compared to unripe, albeit with varying statistical significance (Marvasi et al., 2014).

There is little in the literature surrounding the immune responses of fruiting plants post-harvest. The level to which the tomato fruit's innate immunity to microbial infiltration is compromised post-harvest is not known. However, this provides some insight to the unusual (or lack thereof) trends seen in Fig. 5.

Due to the fact tomatoes were bought in post-harvest state for testing, it is entirely possible that some of the fruits tested were at different stages of ripeness, or had been harvested at different times. This could be the case between var. *alicante* and var. *cerasiforme*, or even between biological replicates of the same tomato variety. This may mean that a tomato that is one week from harvest date may be more susceptible to *Salmonella* proliferation and have less defensive abilities that a tomato one day from harvest.

Tomato maturity can be estimated in part by observing the pigmentation of the tomato as well as firmness of the fruit. Whilst pigmentation does not actually affect the ability of *Salmonella* to proliferate in tomatoes (Marvasi et al., 2014), this estimation is useful for estimating whether tomatoes are at a similar stage of maturity, however not for accurately estimating a date. For future study, it would be advisable to grow the tomatoes from seedlings in controlled laboratory conditions. It would also be wise to measure proliferation of *Salmonella* but most importantly hydrogen peroxide concentration in pre and post-harvest tomatoes. This would provide better insight into the response to microbial invasion that tomato fruits have, post-harvest.

### 4.4.2 Hydrogen Peroxide Quantification Methods

Following the measurement and comparison of hydrogen peroxide concentrations in var. *alicante* and var. *cerasiforme* in both normal and post-infection tomatoes. It was clear some disparities arose. A further quantification of hydrogen peroxide concentrations in both varieties of tomato was carried out, the results of which was be seen in Fig. 6. The results seen share the same trends observed in the initial hydrogen peroxide quantification tests. There was found to be a significant difference ($p$ = 0.0304) in the concentration of hydrogen peroxide between var. *alicante* and var. *cerasiforme.* On average var. *cerasiforme* was found to have more than double the concentration of hydrogen peroxide in the intercellular fluid than var. *alicante.* For this second round of testing, samples were still deproteinised using 10kD spin columns. However, it was deemed unnecessary to further compare these results to samples that were not deproteinised. This is because data and analysis prior showed there to be no significant difference between samples that were and were not deproteinised.

This second quantification of hydrogen peroxide in both tomato varieties, whilst showing the same trends, showed much higher concentrations overall. Tomato var. *cerasiforme* showed on average 100.32pmol of hydrogen peroxide (Fig. 6) compared against 23.6pmol in the initial tests (Fig. 4). The standard error however shown in Fig. 5 for var. *cerasiforme* is high. This is due to the large variation found within the biological replicates for this tomato variety (See Appendix 1.5). The hydrogen peroxide concentration in var. *alicante* is similarly higher in the second measurement, showing 40.71pmol (Fig. 6) compared against 22.7pmol initially (Fig. 4). It must be stated that for this run, a new standard curve of known hydrogen peroxide concentrations was set up.

Whilst the large variation in hydrogen peroxide quantification between test runs in both var. *alicante* and var. *cerasiforme* does pose some questions, the overall trends are in keeping with the hypothesis. It seems that in all instances *Salmonella*'s ability to proliferate in var. *cerasiforme* is reduced when compared with var. *alicante.* In addition, all instances show that the concentration of hydrogen peroxide is consistently higher in var. *cerasiforme* than in var. *alicante.*

However, these variations in hydrogen peroxide concentrations between test runs, may be as previously mentioned, based on current stage of ripeness of the tomato itself, or possibly due to the post-harvest nature of the test tomatoes. However it may also be due to the method of hydrogen peroxide quantification itself and the nature of the sample. It has been reported that accurate and repeatable methods of ROS quantification from plant tissues is difficult to achieve (Chakraborty et al., 2016). There are number of methods for

quantifying ROS in plant and animal tissues. The most reliable of which is fluorescence based methods, specifically fluorescence enhancement rather than fluorescence quenching (Chakraborty et al., 2016; Zhou et al., 1997). The quantitation kit used in this study was a fluorescence enhancing kit from Abcam®. The probe is listed as OxiRed, however this may also be described as AmplexRed in the literature depending on the supplier. Both are denoted as 10-acetyl-3,7-dihydroxyphenoxazine. In the assay, this probe undergoes a single electron oxidation which releases the fluorescent chemical resorufin. However, the concentration of horseradish peroxidase (the peroxidase that catalyses the oxidation), light and pH all play important roles in this reaction (Towne et al., 2004; Zhao et al., 2012). It has been reported that the fluorescent resorufin can decay into less or non-fluorescent products in particular pH ranges, most prominently in ranges 6.2-7.7. This decay is reportedly due to either the polymerisation of resorufin and de-N-acetylation (Towne et al., 2004).

It has also been reported that buffers in sample preparations may affect the outcome of the assay due some buffers containing soluble proteins or other substances that may interfere with fluorescence detection (Chakraborty et al., 2016). This however was discounted as a potential error in this study as distilled, deionised water was used as a buffer in sample preparation. There is also evidence that suggests resorufin may be produced not necessarily by the catylisation of OxiRed probe, but rather in its exposure to light (Zhao et al., 2012). This too however, was discounted as a source of a potential errors in the assay, as every care was taken to incubate the reaction in places with no light.

Whilst the unknown stage of maturity/ripeness of the tomatoes tested and unknown pH levels of resulting samples from said tomato, may have affected some of the measurements of hydrogen peroxide concentration, trends are clearly seen. In all instances, the concentration of hydrogen peroxide was higher in var. *cerasiforme* than in var. *alicante.* In addition, all instances show that var. *cerasiforme* showed an increased resistance to *Salmonella* proliferation when compared with var. *alicante.*

## 4.5 Hydrogen Peroxide Scavenger & *Salmonella* Proliferation Tests

The data trends shown in both the *Salmonella* proliferation tests as well as hydrogen peroxide quantification has shown some insight into the reasoning behind some tomato varieties ability to resist *Salmonella* proliferation more than others. However, in order to confirm it is indeed increased levels and production of hydrogen peroxide in var. *cerasiforme* that gives it increased resistance to *Salmonella* over var. *alicante,* further tests were carried out. If hydrogen peroxide could be removed from the tomato (or at least at the site of inoculation) then *Salmonella* would theoretically be able to proliferate to a higher degree than it would do in normally. Hydrogen peroxide is baceticidal to *Salmonella* with some studies reporting 99% kill rate with 0.5% and 1% hydrogen peroxide (Unlütürk and Turantaş, 1987). Not only this, but the levels of *Salmonella* proliferation between both varieties would be of similar values. Fig. 7 and Fig. 8 Show the results from a further *Salmonella* proliferation test. These tests however, were carried out using a hydrogen peroxide scavenger and compared against a standard proliferation test. This was done for both var. *cerasiforme* and var. *alicante.*

For this test, two different scavengers were chosen to test; sodium pyruvate and mannitol. Sodium pyruvate is a well-known scavenger of free radicals from ROS like hydrogen peroxide and has even been used as protective agent against in animal disorders caused by ROS like hydrogen peroxide (Jagtap et al., 2003; Salahudeen et al., 1991; Shostak et al., 2000). It has also been reported that mannitol acts as a strong free radical scavenger (Silvestre et al., 2008).

### 4.5.1 Mannitol

Analysis shows that there lies no significant difference ($p$ = 0.182) between the level of *Salmonella* proliferation between control and mannitol treatments in var. *alicante.* These results are however, unexpected. Whilst the level of hydrogen peroxide in var. *alicante* has been established as consistently lower than that of var. *cerasiforme*, it would still be expected that with the scavenging ability of mannitol would allow *Salmonella* to proliferate to a higher degree. The expectation of this difference to be statistically significant was not known, however it should be stated that whilst not statistically significant, the increase in *Salmonella* proliferation was higher in the mannitol treatment than the control for var. *alicante.*

The trend observed for var. *cerasiforme* was however, somewhat different. It was found that a significant difference ($p$ = 0.013) lied in the increase in *Salmonella* proliferation between control and mannitol treatments in var. *cerasiforme,* with the greater of the two being seen in the mannitol treatment. This particular trend was in keeping with the aforementioned hypothesis. It would be expected that providing mannitol is scavenging hydrogen peroxide at the site of inoculation then the barriers for increased *Salmonella* proliferation would be (at least in part) removed.

It would also stand to reason that the greatest difference seen would be between control and mannitol treatments in var. *cerasiforme* rather than var. *alicante.* If the greatest barrier to microbial infiltration and *Salmonella* proliferation in the tomatoes is the concentration of hydrogen peroxide, then there should be an increase in *Salmonella* proliferation when this barrier is removed. If it is also conceded that var. *cerasiforme* contains the highest amount/largest production of hydrogen peroxide, then it would follow that the largest difference in *Salmonella* proliferation between control and mannitol treatments would be seen in var. *cerasiforme.* The data presented in Fig. 7 supports this conclusion.

As can be seen in Fig. 6 in the control data, there lies a significant difference ($p$ = 0.0082) in the ability of *Salmonella* to proliferate in var. *cerasiforme* compared with var. *alicante,* with *Salmonella* proliferating less in var. *cerasiforme.* This trend is in keeping with all other previous proliferation tests in this study, as well as the ones reported in Marvasi et al. (2014). However, there also lies a significant difference ($p$ = 0.016) in *Salmonella* proliferation between var. *cerasiforme* and var. *alicante* in the mannitol treatments. It should be noted however that the strength of the significance is weaker in mannitol treatment, and the estimated difference is also smaller (see Table. 2).

Further analysis was carried to test if the estimated difference in *Salmonella* proliferation between var. *cerasiforme* and var. *alicante* in the control was significantly different from the estimated difference in *Salmonella* proliferation between var. *cerasiforme* and var. *alicante* in the mannitol treatment (Table. 2). For this 2-sample t-test, the estimated differences were used as means and the pooled standard deviation from the original t-tests used as the standard deviation. The result found that there was no significant difference ($p$ = 0.057) between the estimated differences of either test. Whilst this overall was an unexpected result, it is in keeping with the previous statistical tests. Whilst the difference between estimated differences is not significant, the estimated difference in the mannitol treatment is smaller than in the control group (Table. 2). This does suggest in part that the scavenging ability of mannitol produces levels of *Salmonella* proliferation closer to one another between tomato varieties, than would be seen in the control group.

### 4.5.2 Sodium Pyruvate

In order to further confirm the hypothesis that hydrogen peroxide is one of the major sources of difference in *Salmonella* resistance between var. *cerasiforme* and var. *alicante,* a second scavenger was tested.

The trends seen in the results for the sodium pyruvate scavenger tests are different to those seen in the mannitol tests. Analysis showed that there existed no significant difference in *Salmonella* proliferation between control and sodium pyruvate treatment, for both tomato varieties (var. *alicante* – $p$ = 0.720, var. *cerasiforme* – $p$ = 0.193). These data are not representative of the original hypothesis. Whilst the increase in *Salmonella* proliferation in var. *cerasiforme* does increase with the sodium pyruvate treatment, the difference is not significant.

However, when analysing the increase in *Salmonella* proliferation between var. *cerasiforme* and var. *alicante* in the control group, there lies a significant difference ($p$ = <0.000). This result is again, in keeping with the original hypothesis. Var. *cerasiforme* is consistently more resistant to *Salmonella* proliferation than var. *alicante.* However, unusually, there also lies a significant difference ($p$ = <0.000) in *Salmonella* proliferation between var. *cerasiforme* and var. *alicante* in the sodium pyruvate treatment. This trend was also seen in the mannitol treated tomatoes, and is unexpected.

Similarly with the mannitol treated tomatoes, further analysis was carried out with the sodium pyruvate treated tomatoes to see if the estimated difference in *Salmonella* proliferation between var. *cerasiforme* and var. *alicante* in the control was significantly different from the estimated difference in *Salmonella* proliferation between var. *cerasiforme* and var. *alicante* in the mannitol treatment (Table. 3). Analysis showed there was no significance difference ($p$ = 0.169) between the estimated differences of *Salmonella* proliferation in sodium pyruvate treated tomatoes (var. *cerasiforme* and var. *alicante*) and sodium pyruvate treated tomatoes (var. *cerasiforme* and var. *alicante*).

The aforementioned results and subsequent analysis however, does pose some questions regarding the efficacy of mannitol and sodium pyruvate as a scavengers. There are currently no reports regarding the use of mannitol or sodium pyruvate as a free radical scavenger in tomato tissues, however there are mixed reports from animal based studies. It has been reported that mannitol acts as an effective free radical scavenger when attenuating gentamicin toxicity (Song and Schacht, 1996). However, other papers have suggested that mannitol is not as effective as other free radical scavengers (Mayo et al., 2003). These reports however are based from mammalian models and may not be directly comparable to plant tissues. In addition to this sodium pyruvate has been reported to be

an efficient scavenger of hydrogen peroxide (Ramakrishnan et al., 2016). Although this example is also based of mammalian cellular environments.

Nevertheless, it may not be possible to remove 100% of hydrogen peroxide from the site of inoculation especially if mannitol is not a consistently effective scavenger. This means that creating an environment in the tomato free of hydrogen peroxide for *Salmonella* to grow is difficult. The tomatoes were treated with mannitol at the site of inoculation (see section 2.4), however the site of inoculation is not necessarily representative of the tomato as a whole. Hydrogen peroxide, as well as providing important immune capabilities in tomatoes, also plays an important role as a messenger molecule (Río and Puppo, 2009). It has been reported that hydrogen peroxide can act as a local signalling molecule and even act as a diffusible signal for cells close the site of wounding or a microbial attack, inducing immune responses described previously such as micro-bursts of hydrogen peroxide or transcription of defensive genes (Alvarez et al., 1998; Orozco-Cárdenas et al., 2001). This means that whilst the tomatoes were periodically treated with mannitol and sodium pyruvate, and *Salmonella* continued to proliferate in the tomato, cell signalling may have caused production of hydrogen peroxide at neighbouring cells. This becomes especially important when considering the methodology of measuring increase in *Salmonella* proliferation, as the whole tomato is homogenised before plating on XLD (see section 2.1).

In addition to this the concentration of both scavengers should be considered. Finding an appropriate concentration of scavenger to use in the aforementioned scavenger tests, can be difficult and depend entirely on the context of use. For example, some studies have used mannitol in concentrations of 20mmol/L when using it in cardioplegia treatments (Larsen et al., 2002). Whereas some studies regarding the pharmaceutical uses of mannitol have used concentrations such as 1M (Silvestre et al., 2008). This is further complicated by two factors. Firstly, the concentrations of hydrogen peroxide within var. *cerasiforme* and var. *alicante* can vary. This means the availability of mannitol and sodium pyruvate to scavenge all hydrogen peroxide will be different. Secondly is varying efficacy of both mannitol and sodium pyruvate that has been previously mentioned. For future study, it would be ideal to first test the efficacy of each scavenger. This may be done by measuring the concentration of hydrogen peroxide dissolved in water before and after treatment with varying concentrations of mannitol and sodium pyruvate.

# 4.6 Hydrogen Peroxide Scavenger Growth Curve

Whilst the trends present in the scavenger proliferation tests were not always consistent, there was indication that when hydrogen peroxide is removed from the tomato though the scavenging properties of mannitol or sodium pyruvate, there was an increase in overall proliferation of *Salmonella*. However, in order to confirm this conclusion further variables had to be considered. To ensure that the results presented in Fig. 7 + 8 were accurately portraying the increase in growth of *Salmonella* in a hydrogen peroxide free environment, there had to be confirmation that the addition of the hydrogen peroxide scavengers, mannitol and sodium pyruvate, were not affecting the growth of *Salmonella* in any way.

Mannitol is a sugar alcohol which has multiple uses across different disciplines. It is used as sweetener in foods but also in the medical industry as treatment for brain injuries (VARZAKAS, 2016; Wakai et al., 2013). In microbiology, it is one of the main composites of Mannitol Salt Agar (MSA), a common selective and differential agar medium often used in clinical settings to distinguish pathogenic microorganisms (Willey et al., 2011). Its purpose in MSA is to distinguish microorganisms that can ferment mannitol. However, it is also a sugar and has the potential to act as a source of energy for certain microorganisms. There have even been reports that mannitol may enhance biofilm formation in *Salmonella* (Ngwai et al., 2006).

Sodium pyruvate also has the potential to affect the viability of *Salmonella* to proliferate in the tomato. There have been a number of studies that show that sodium pyruvate can act as a growth inducer for microorganisms (Morishige et al., 2013). Hydrogen peroxide in some instances has been used an antimicrobial and can cause enteric bacteria like *Salmonella* to enter ‘viable but non-culturable’ state (VBNC) where the cells are metabolically active but not necessarily culturable on media (Xu et al., 1982). It has subsequently been shown that addition of sodium pyruvate can ‘resurrect’ *Salmonella* from a hydrogen peroxide treated VBNC state (Morishige et al., 2013).

Due to the effects both mannitol and sodium pyruvate have been shown to have on microorganisms, it was necessarily to perform 14.5 hour growth curves, treated with both mannitol and sodium pyruvate respectively. The data for this can be seen in Fig. 9. This data shows the growth of *Salmonella* over a 14.5 hour period. *Salmonella* was treated with mannitol, sodium pyruvate and PBS (control) respectively. Clear and distinct phases of microbial growth can be seen in Fig. 9. The most prominent of which being the lag and

exponential phase. In a model image of microbial growth the lag, exponential, stationary and death phase would be seen. However, this standard four phase growth curve of any bacteria is not entirely represented here. This is of little consequence however as for the purpose of this experiment the exponential phase was the most important and would be the most telling. It can be seen in Fig. 9, that the growth lines representing mannitol and control are almost identical throughout the 14.5 hour growth period. Sodium pyruvate is also similar to the mannitol treatment and control.

Statistically testing for significance between three curvilinear sets of data over multiple data points is difficult and so the data presented in Fig. 9 was altered to allow for statistical testing. The data shown in Fig. 10 is a linearised version of the data in Fig. 9. To achieve this data points from hour 8 to hour 14 were isolated and plotted into a scatter plot. This was done for mannitol and sodium pyruvate treated as well as control. Linear regression allowed for the generation of coefficients, standard error as well as equations describing the slope of each trend line. These data was then used to test for significant difference between each treatment and the control.

Analysis showed that there was no significant difference ($p = 0.884$) in the growth of *Salmonella* over time in the sodium pyruvate treatment and the control. This data therefore indicates that the addition of sodium pyruvate in the scavenger proliferation tests had no significant effect, be it positive or negative, on the ability of *Salmonella* to proliferate in either tomato variety. However, analysis showed that there was a significant difference ($p = 0.003$) in the growth of *Salmonella* over time between the mannitol treatment and the control. This analysis suggests that mannitol did have an effect of the ability of *Salmonella* to proliferate in either tomato variety.

However, the level of significance and subsequent $p$ values resulting from this analysis may be of less importance when considering the estimates for difference and $r^2$ values in these linear regressions. Table. 3 shows the line equations and $r^2$ values for each treatment. Each of the $r^2$ values indicates that the exponential phase of *Salmonella* growth in mannitol, sodium pyruvate treatment and control fit a straight line almost exactly. This means that any variation between mannitol, sodium pyruvate treatment or control, will likely be statistically significant. The importance of the statistical significance lessens further when considering the estimates for difference in each test. The estimated difference between the mannitol treatment and the control was 0.00413, and the estimated difference between the sodium pyruvate treatment and the control was -0.00018. These estimates are small enough to conclude that (statistically significant or

not) the addition of mannitol or sodium pyruvate to the scavenger proliferation tests, did not help nor hinder *Salmonella* growth to a degree worthy of note.

## 4.7 RNA Extraction

The data and subsequent analysis in this study has established that on average, var. *cerasiforme* is consistently more resistant to *Salmonella* proliferation than var. *alicante.* In addition, var. *cerasiforme* consistently shows a higher concentration of hydrogen peroxide in its intercellular space than var. *alicante.*

As mentioned in section 1.6, *Salmonella* also contains a number of genes that are involved in combating hydrogen peroxide induced oxidative stress. However the regulation and expression of these genes in proliferation in tomatoes has not been studied. The initial aim of this experiment was to use qPCR to quantify the relative expression of these genes after inoculation and incubation in var. *cerasiforme* and var. *alicante.* The first step was to extract RNA from *Salmonella.*

RNA extraction has become increasingly more viable with the advent of RNA extraction kits. However, whilst this is relatively simple to do on bacteria grown in laboratory media, it is a harder task to do so from biological environments. Obtaining accurate representations of bacterial transcriptomes can be difficult if extraction of high yield RNA with low DNA and protein content is also a challenge (Heera et al., 2015). It was important that *Salmonella* RNA was extracted after incubation within the tomato, so that the gene response to hydrogen peroxide induced oxidative stress could be measured.

The results from the RNA extraction can be seen in Table. 5. What is immediately noticeable is that for all extractions of *Salmonella* RNA from incubation in var. *cerasiforme* or var. *alicante,* is very low. The highest concentration of RNA extracted from *Salmonella* was 5.6ng/µl. Expected yield of RNA via extraction kits can vary greatly depending on the cell type and medium in which the cell resides (Heera et al., 2015; Schwochow et al., 2012). RNA extractions from saliva samples for example have reported yields as high as 0.89 – 7.1µg (Pandit et al., 2013). It has also been reported that whole blood can be an appropriate source of RNA, which papers noting RNA extraction yields of 1.4 - 18µg (Schwochow et al., 2012).

Most examples in the literature report much higher yields from RNA extraction than is present in Table. 5. This initially posed problems when considering moving forward with qPCR. As well as overall yield, purity of extractions had to be considered. Samples

considered pure normally show a A1260/A230 ratio of 2.0-2.2 A second indication of purity is represented by A260/A280 ratio, pure samples are considered to have a A260/A280 ratio of 1.8-2.0 (NanoDrop Technologies™, 2007). All samples (bar one) showed extractions to be impure.

There are many potential reasons why extractions such as those represented in Table. 5 should show such low yields and impurity. One common reason is the contamination of DNA. Often RNA extraction kits utilise DNase activity along with spin columns to ensure that as little DNA as possible ends up in the final product. However, many reports indicate that accidental extraction of DNA in the final extraction product is sometimes unavoidable (Heera et al., 2015; Schwochow et al., 2012).

It is also possible that the environment in which the cells were extracted from affected the resulting extraction products. The methodology used to extract *Salmonella* from the tomato (see section 2.6) did not entirely account for the removal of all tomato cells and/or tissue. The pelleted cells that were used for extraction may have potentially contained some plant tissue. If this were the case then the use of a standard RNA extraction kit would not be effective on plant tissue. Not only does plant tissue require specialised RNA extraction kits, it is also notoriously difficult to extract high yield RNA (Ouyang et al., 2014). It is also possible that in addition to potential leftover plant tissue/cells, the resulting cells prepared for extraction also contained RNase's. It has been shown that wild tomato species often produce particular glycoproteins extracellularly. These glycoproteins often have RNase activity (McClure et al., 1989; Parry et al., 1998). If these glycoproteins were not fully removed prior to extraction, they could have broken down any RNA present in the samples, potentially resulting in low yields.

Due to time constraints, troubleshooting of the RNA extractions described here, nor the subsequent qPCR, did not take place. However, suggestions for future research and the limitations of this current study are described in section 4.8.

## 4.8 Limitations & Future Research

Following analysis of the results presented in this study, it is clear that whilst some of the data shows clear support for the overarching aims, others will require further research. In this section, the limitations of some experiments as well as potential for further research is discussed.

### 4.8.1 Pre – Harvest Hydrogen Peroxide Quantification

As mentioned previously, all tomatoes used and tested in this study were bought and not growth from seedlings in the laboratory. This posed questions regarding the ability of tomatoes to respond effectively to biotic stressors such as *Salmonella* infection. Plants immune responses are compromised in a post-harvest state (Shi et al., 2007). It would therefore follow that the tomatoes ability to respond to infection by producing hydrogen peroxide may also be hindered. In order to confirm this, further tests could be carried out. The data in Fig. 4 indicated that there was limited difference between the concentrations of hydrogen peroxide in var. *cerasiforme* and var. *alicante* post infection compared to no infection. This however, may not be the case in tomatoes that are pre-harvest. Here we suggest repeating experiments laid out in section 2.3 however on pre-harvest tomatoes. This may show the differences in immune responses in var. *cerasiforme* and var. *alicante* between pre and post-harvest states.

### 4.8.2 Tomato Ripeness

As previously mentioned the cellular environment of a tomato differs greatly depending on the stage of ripeness. For example unripe tomatoes are significantly more acidic than ripe tomatoes (Arias et al., 2000; Asplund and Nurmi, 1991). Not only this, but studies show that ripe tomatoes are on average more conducive to *Salmonella* proliferation than unripe tomatoes (Marvasi et al., 2014). These are some suggestions for the variation in *Salmonella* proliferation seen in the various tests in this study. To remedy this, we suggest growing all tomato varieties from seedlings in controlled laboratory conditions. Stage of maturity and ripeness can be kept constant by harvesting all tomatoes at a given time.

### 4.8.3 Scavenger Efficacy

One of the potential issued mentioned in section 4.5 was regarding the efficacy of mannitol and sodium pyruvate as hydrogen peroxide scavengers. It also meant choosing appropriate concentrations to use of said scavengers was difficult. To remedy this, we suggest carrying out a further test to measure the efficacy of each scavenger. This may be done by treating known concentrations of hydrogen peroxide with varying concentrations of scavenger and measuring the levels of hydrogen peroxide before and after treatment.

### 4.7.4 qPCR and Genetic Anaiysls

Time constraints regrettably meant that genetic analysis of genes present in *Salmonella* and *S. lycopersicum* was unable to be carried out. There are however genes reported in both organisms responsible for immune responses. The *RBOH1* codes for NADPH oxidase in *S. lycopersicum.* This gene is able to regulate the production of hydrogen peroxide in tomatoes, and certain stressors have been shown to cause upregulation of this gene in tomatoes (Mei et al., 2017). However, the relative level of expression of this gene in different varieties of tomatoes is not yet known. This study has established that some varieties of tomato consistently produce higher amounts of hydrogen peroxide, specifically var. *cerasiforme* over var. *alicante.* It would therefore follow that *RBOH1* may be expressed to a higher degree in a tomato such as var. *cerasiforme.* Therefore, we suggest qPCR analysis of the *RBOH1* gene in var. *cerasiforme* and var. *alicante.*

In addition to this, *Salmonella* has multiple genes involved in combatting oxidative stress. These genes code for enzymes that are either catalases. These catalases are coded for by the KatE, KatN (part of Rpos regulon) and KatG (part of OxyR regulon) genes (Ibanez-Ruiz et al., 2000; Morgan et al., 1986). In addition, *Salmonella* also contains peroxiredoxin proteins, which are able to scavenge hydrogen peroxide with the use of a NADH or NADPH catalytic substrate (Horst et al., 2010). These peroxiredoxins are coded for by AhpC and AhpF (part of the OxyR regulon). Studies have shown that both types of protein are responsible for the primary response to oxidative stress in *Salmonella,* with deletion mutants of Ahp and Kat genes showing greatly reduced ability of scavenge endogenous hydrogen peroxide (Seaver and Imlay, 2001). This demonstrates that the plant – pathogen response is a complex and dynamic system.

As a continuation of the results shown in Table. 5, we suggest qPCR analysis of the genes KatE, KatN, KatG, AhpC and AhpF in *Salmonella*. We suggest this be done from cells extracted from a tomato inoculation as well as in laboratory media.

# 5. C O N C L U S I O N S

The data in this study suggests that there lies an inherent biological susceptibility to *Salmonella* proliferation in tomatoes, but more importantly that this susceptibility varies between varieties of tomato. This study has demonstrated specifically that *Solanum lycopersicum* var. *cerasiforme* is consistently more resistant to *Salmonella* proliferation than var. *alicante.* This difference is echoed in other varieties studied in the literature, suggesting that cherry varieties are more resistant to *Salmonella* proliferation than other, larger varieties. This study has shown strong indication that this difference is due to the amount of hydrogen peroxide produced by these tomato varieties. Tomato variety *cerasiforme* was consistently shown to produce significantly more hydrogen peroxide into the intercellular space than var. *alicante.* This finding was enabled by a novel method of intercellular fluid extraction in tomato fruits. Unusual trends were observed when measuring the concentration of hydrogen peroxide in both tomato varieties post infection of *Salmonella.* No significant increase in the immune response of either tomato variety was observed when being analysed after *Salmonella* infection. This study suggests however, this trend is due to methodological errors and that further research should be carried out to confirm this. Time constraints meant that proper genetic analysis of the genes involved in the tomato or *Salmonella* immune responses, was not possible. However, identification of potential genes responsible for said immune responses, allowed for suggestion of further research. This study has reiterated that the plant-pathogen immune responses are complex and dynamic with a host of confounding variables. Whilst this study has demonstrated hydrogen peroxide concentration is clearly a factor that contributes to the susceptibility of tomatoes to *Salmonella* proliferation, there multiple facets to consider such as pre/post-harvest nature of the fruit, tomato ripeness as well as the multitude of stress response genes present in *Salmonella.*

# 6. B I B L I O G R A P H Y

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# A P P E N D I X

This appendix contains full, unaltered data from the results shown in this study. All analyses of the data as referred to in the text is also shown. Appendices are shown in chronological order in reference to the results.

## Appendix 1 – Raw data

**Appendix 1.1.** Increase in *S.* Typhimurium (4028) Proliferation (log) in *S. lycopersicum* var. *Alicante* and var. *cerasiforme*

| | Biological Replicates | CFU | | | Increase in *Salmonella* Proliferation (log) |
|---|---|---|---|---|---|
| | | Tech Rep 1 | Tech Rep 2 | Average | |
| Alicante | 1 | 39 | 96 | 67.5 | 3.294 |
| | 2 | 60 | 88 | 74 | 3.322 |
| | 3 | 81 | 76 | 78.5 | 3.371 |
| | 4 | 217 | 149 | 183 | 3.652 |
| | 5 | 300 | 300 | 300 | 3.942 |
| | 6 | 300 | 300 | 300 | 3.760 |
| **Average** | | | | | **3.557** |
| | | | | | |
| Cherry | 1 | 136 | 138 | 137 | 3.226 |
| | 2 | 28 | 20 | 24 | 2.469 |
| | 3 | 5 | 4 | 4.5 | 1.735 |
| | 4 | 12 | 14 | 13 | 2.203 |
| | 5 | 49 | 63 | 56 | 2.830 |
| | 6 | 105 | 117 | 111 | 3.127 |
| **Average** | | | | | **2.598** |

**Appendix 1.2** $H_2O_2$ concentration from deproteinised vs. non-deproteinised samples of *S. lycopersicum* var. *Alicante* intercellular fluid

| | **$H_2O_2$ (pmol)** | | | |
|---|---|---|---|---|
| | Deproteinised Samples | | Non-deproteinised Samples | |
| | Biological Replicate 1 | Biological Replicate 2 | Biological Replicate 1 | Biological Replicate 2 |
| Technical Replicate 1 | 26.9 | 23.1 | 21.2 | 24.1 |
| Technical Replicate 2 | 26.5 | 23.1 | 20.8 | 24.6 |
| **Average** | **26.7** | **23.1** | **21** | **24.4** |

**Appendix 1.3** $H_2O_2$ concentration from deproteinised vs. non-deproteinised samples of *S. lycopersicum* var. *cerasiforme* intercellular fluid

| | **$H_2O_2$ (pmol)** | | | | | |
|---|---|---|---|---|---|---|
| | DEPROTEINISED SAMPLES | | | NON-DEPROTEINISED SAMPLES | | |
| | BIOLOGICAL REPLICATE 1 | BIOLOGICAL REPLICATE 2 | BIOLOGICAL REPLICATE 3 | BIOLOGICAL REPLICATE 1 | BIOLOGICAL REPLICATE 2 | BIOLOGICAL REPLICATE 3 |
| TECHNICAL REPLICATE 1 | 22.97 | 23.62 | 19.09 | 22.32 | 28.14 | 22.32 |
| TECHNICAL REPLICATE 2 | 21.03 | 24.26 | 22.32 | 21.03 | 27.49 | 20.39 |
| **AVERAGE** | **22** | **23.9** | **20.7** | **21.6** | **27.8** | **21.3** |

**Appendix 1.4** Intercellular concentration of $H_2O_2$ in *S. lycopersicum* var. *alicante* and var. *cerasiforme* post infection of *S.* Typhimurium

| | **$H_2O_2$ (pmol)** | | | | | |
|---|---|---|---|---|---|---|
| | CHERRY | | | ALICANTE | | |
| | BIOLOGICAL REPLICATE 1 | BIOLOGICAL REPLICATE 2 | BIOLOGICAL REPLICATE 3 | BIOLOGICAL REPLICATE 1 | BIOLOGICAL REPLICATE 2 | BIOLOGICAL REPLICATE 3 |
| TECHNICAL REPLICATE 1 | 19 | 19 | 19 | 9.4 | 6.8 | 4.8 |
| TECHNICAL REPLICATE 2 | 19 | 19.7 | 19.7 | 9.4 | 6.8 | 5.5 |
| **AVERAGE** | **19** | **19.35** | **19.35** | **9.4** | **6.8** | **5.2** |

**Appendix 1.5** Intercellular production of $H_2O_2$ (pmol) in *S. lycopersicum* var. *alicante* and var. *cerasiforme*

| | **$H_2O_2$ (pmol)** | | | | |
|---|---|---|---|---|---|
| **TOMATO** | REPLICATE 1 | REPLICATE 2 | REPLICATE 3 | REPLICATE 4 | AVERAGE |
| CHERRY | 58.137 | 59.317 | 89.281 | 194.548 | **100.321** |
| ALICANTE | 31.909 | 42.839 | 39.661 | 48.453 | **40.716** |

**Appendix 1.6** Increase in *S.* Typhimurium (LT2 14028) Proliferation (log) in *S. lycopersicum* var. *alicante* and var. *cerasiforme* when treated with Mannitol vs. control

| | Biological Replicates | CFU | | | Increase in *Salmonella* Proliferation (log) |
|---|---|---|---|---|---|
| | | Tech Rep 1 | Tech Rep 2 | Average | |
| Alicante (Mannitol Treatment) | 1 | 176 | 131 | 153.5 | 5.286 |
| | 2 | 237 | 224 | 230.5 | 5.507 |
| | 3 | 109 | 104 | 106.5 | 5.082 |
| | 4 | 330 | 335 | 332.5 | 5.632 |
| | 5 | 164 | 108 | 136 | 5.254 |
| | 6 | 104 | 113 | 108.5 | 5.144 |
| **Average** | | | | | **5.317** |
| Alicante (Control) | 1 | 173 | 123 | 148 | 5.287 |
| | 2 | 134 | 68 | 101 | 5.144 |
| | 3 | 227 | 194 | 210.5 | 5.445 |
| | 4 | 15 | 84 | 49.5 | 4.854 |
| | 5 | 56 | 130 | 93 | 5.135 |
| | 6 | 107 | 59 | 83 | 4.994 |
| **Average** | | | | | **5.143** |
| Cherry (Mannitol Treatment) | 1 | 461 | 560 | 510.5 | 5.226 |
| | 2 | 245 | 374 | 309.5 | 5.006 |
| | 3 | 208 | 289 | 248.5 | 4.909 |
| | 4 | 593 | 258 | 425.5 | 5.143 |
| | 5 | 170 | 177 | 173.5 | 4.755 |
| | 6 | 361 | 172 | 266.5 | 4.927 |
| **Average** | | | | | **4.994** |
| Cherry (Control) | 1 | 239 | 222 | 230.5 | 4.892 |
| | 2 | 129 | 153 | 141 | 4.653 |
| | 3 | 236 | 146 | 191 | 4.807 |
| | 4 | 113 | 160 | 136.5 | 4.658 |
| | 5 | 37 | 11 | 24 | 3.913 |
| | 6 | 164 | 113 | 138.5 | 4.690 |
| **Average** | | | | | **4.602** |

**Table. 1.7** Increase in *S.* Typhimurium (LT2 14028) Proliferation (log) in *S. lycopersicum* var. *alicante* and var. *cerasiforme* when treated with Sodium Pyruvate vs. control

| | Biological Replicates | CFU | | | Increase in *Salmonella* Proliferation (log) |
|---|---|---|---|---|---|
| | | Tech Rep 1 | Tech Rep 2 | Average | |
| Alicante (Sodium Pyruvate Treatment) | 1 | 191 | 152 | 171.5 | 4.887 |
| | 2 | 82 | 73 | 77.5 | 4.562 |
| | 3 | 113 | 97 | 105 | 4.704 |
| | 4 | 212 | 202 | 207 | 4.989 |
| | 5 | 184 | 181 | 182.5 | 4.930 |
| | 6 | 35 | 120 | 77.5 | 4.558 |
| **Average** | | | | | **4.772** |
| Alicante (Control) | 1 | 159 | 136 | 147.5 | 4.505 |
| | 2 | 205 | 251 | 228 | 5.092 |
| | 3 | 202 | 111 | 156.5 | 4.920 |
| | 4 | 133 | 106 | 119.5 | 4.734 |
| | 5 | 148 | 165 | 156.5 | 4.862 |
| | 6 | 103 | 143 | 123 | 4.764 |
| **Average** | | | | | **4.813** |
| Cherry (Sodium Pyruvate Treatment) | 1 | 210 | 158 | 184 | 4.374 |
| | 2 | 92 | 100 | 96 | 4.105 |
| | 3 | 124 | 151 | 137.5 | 4.254 |
| | 4 | 85 | 106 | 95.5 | 4.096 |
| | 5 | 69 | 65 | 67 | 3.929 |
| | 6 | 99 | 110 | 104.5 | 4.135 |
| **Average** | | | | | **4.149** |
| Cherry (Control) | 1 | 64 | 115 | 89.5 | 4.074 |
| | 2 | 88 | 191 | 139.5 | 4.227 |
| | 3 | 69 | 79 | 74 | 3.966 |
| | 4 | 54 | 72 | 63 | 3.909 |
| | 5 | 92 | 104 | 98 | 4.094 |
| | 6 | 43 | 107 | 75 | 3.971 |
| **Average** | | | | | **4.041** |

**Appendix 1.8** Growth of *Salmonella* in treatment of mannitol, sodium pyruvate and control.

| | Mannitol Treatment | | | | | | Control (PBS) | | | | | | Sodium Pyruvate Treatment | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C | A | | B | | C | | A | | B | | C | | A | | B | | C | |
| 1 | 0.224 | 0.28025 | 0.312 | 0.32 | 0.288 | 0.327 | 0.24 | 0.31275 | 0.264 | 0.32525 | 0.274 | 0.3025 | 0.255 | 0.30575 | 0.243 | 0.284 | 0.238 | 0.28425 |
| 1 | 0.285 | | 0.321 | | 0.343 | | 0.351 | | 0.358 | | 0.287 | | 0.329 | | 0.282 | | 0.285 | |
| 1 | 0.305 | | 0.346 | | 0.36 | | 0.347 | | 0.377 | | 0.344 | | 0.333 | | 0.307 | | 0.313 | |
| 1 | 0.307 | | 0.301 | | 0.317 | | 0.313 | | 0.302 | | 0.305 | | 0.306 | | 0.304 | | 0.301 | |
| 2 | 0.067 | 0.06925 | 0.07 | 0.06475 | 0.064 | 0.063 | 0.069 | 0.0645 | 0.069 | 0.0665 | 0.066 | 0.06375 | 0.068 | 0.06425 | 0.067 | 0.06475 | 0.067 | 0.065 |
| 2 | 0.068 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.064 | | 0.065 | |
| 2 | 0.064 | | 0.063 | | 0.063 | | 0.063 | | 0.071 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | |
| 2 | 0.078 | | 0.063 | | 0.062 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.065 | | 0.065 | |
| 3 | 0.083 | 0.07275 | 0.069 | 0.064 | 0.064 | 0.06225 | 0.069 | 0.0635 | 0.067 | 0.065 | 0.065 | 0.063 | 0.067 | 0.0635 | 0.066 | 0.06325 | 0.066 | 0.0645 |
| 3 | 0.068 | | 0.062 | | 0.061 | | 0.061 | | 0.062 | | 0.062 | | 0.062 | | 0.063 | | 0.065 | |
| 3 | 0.063 | | 0.062 | | 0.062 | | 0.062 | | 0.07 | | 0.063 | | 0.062 | | 0.061 | | 0.063 | |
| 3 | 0.077 | | 0.063 | | 0.062 | | 0.062 | | 0.061 | | 0.062 | | 0.063 | | 0.063 | | 0.064 | |
| 4 | 0.084 | 0.073 | 0.069 | 0.0635 | 0.063 | 0.06225 | 0.07 | 0.06375 | 0.067 | 0.065 | 0.065 | 0.06275 | 0.067 | 0.06375 | 0.065 | 0.0635 | 0.065 | 0.06425 |
| 4 | 0.067 | | 0.061 | | 0.062 | | 0.062 | | 0.062 | | 0.062 | | 0.062 | | 0.063 | | 0.065 | |
| 4 | 0.063 | | 0.062 | | 0.062 | | 0.062 | | 0.069 | | 0.062 | | 0.063 | | 0.062 | | 0.063 | |
| 4 | 0.078 | | 0.062 | | 0.062 | | 0.061 | | 0.062 | | 0.062 | | 0.063 | | 0.064 | | 0.064 | |
| 5 | 0.078 | 0.072 | 0.069 | 0.0635 | 0.063 | 0.0625 | 0.07 | 0.06425 | 0.066 | 0.06475 | 0.064 | 0.0625 | 0.068 | 0.0635 | 0.066 | 0.06375 | 0.064 | 0.064 |
| 5 | 0.068 | | 0.061 | | 0.062 | | 0.062 | | 0.062 | | 0.061 | | 0.062 | | 0.063 | | 0.065 | |
| 5 | 0.064 | | 0.062 | | 0.063 | | 0.062 | | 0.07 | | 0.063 | | 0.062 | | 0.062 | | 0.063 | |
| 5 | 0.078 | | 0.062 | | 0.062 | | 0.063 | | 0.061 | | 0.062 | | 0.062 | | 0.064 | | 0.064 | |
| 6 | 0.077 | 0.07925 | 0.07 | 0.06525 | 0.064 | 0.06375 | 0.071 | 0.0655 | 0.068 | 0.066 | 0.066 | 0.064 | 0.069 | 0.06525 | 0.066 | 0.06475 | 0.066 | 0.06575 |
| 6 | 0.069 | | 0.064 | | 0.064 | | 0.064 | | 0.063 | | 0.063 | | 0.064 | | 0.064 | | 0.067 | |
| 6 | 0.065 | | 0.064 | | 0.064 | | 0.064 | | 0.07 | | 0.064 | | 0.064 | | 0.063 | | 0.064 | |
| 6 | 0.106 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.064 | | 0.066 | | 0.066 | |
| 7 | 0.076 | 0.079 | 0.069 | 0.06425 | 0.064 | 0.06275 | 0.068 | 0.06375 | 0.067 | 0.06525 | 0.065 | 0.063 | 0.068 | 0.06425 | 0.066 | 0.06425 | 0.065 | 0.065 |
| 7 | 0.069 | | 0.062 | | 0.062 | | 0.062 | | 0.063 | | 0.062 | | 0.062 | | 0.064 | | 0.066 | |
| 7 | 0.065 | | 0.063 | | 0.063 | | 0.063 | | 0.069 | | 0.063 | | 0.063 | | 0.062 | | 0.064 | |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 | 0.106 | | 0.063 | | 0.062 | | 0.062 | | 0.062 | | 0.062 | | 0.064 | | 0.065 | | 0.065 | |
| 8 | 0.082 | 0.07425 | 0.07 | 0.065 | 0.065 | 0.06375 | 0.07 | 0.065 | 0.067 | 0.06575 | 0.065 | 0.0635 | 0.069 | 0.065 | 0.066 | 0.0645 | 0.065 | 0.0675 |
| 8 | 0.069 | | 0.063 | | 0.064 | | 0.063 | | 0.064 | | 0.063 | | 0.063 | | 0.064 | | 0.076 | |
| 8 | 0.066 | | 0.063 | | 0.063 | | 0.064 | | 0.07 | | 0.063 | | 0.064 | | 0.063 | | 0.064 | |
| 8 | 0.08 | | 0.064 | | 0.063 | | 0.063 | | 0.062 | | 0.063 | | 0.064 | | 0.065 | | 0.065 | |
| 9 | 0.08 | 0.074 | 0.07 | 0.06525 | 0.064 | 0.0635 | 0.071 | 0.06525 | 0.067 | 0.066 | 0.066 | 0.06425 | 0.069 | 0.06525 | 0.066 | 0.06525 | 0.065 | 0.065 |
| 9 | 0.069 | | 0.063 | | 0.063 | | 0.063 | | 0.064 | | 0.063 | | 0.064 | | 0.065 | | 0.066 | |
| 9 | 0.066 | | 0.064 | | 0.064 | | 0.064 | | 0.07 | | 0.064 | | 0.064 | | 0.064 | | 0.064 | |
| 9 | 0.081 | | 0.064 | | 0.063 | | 0.063 | | 0.063 | | 0.064 | | 0.064 | | 0.066 | | 0.065 | |
| 10 | 0.079 | 0.074 | 0.07 | 0.065 | 0.064 | 0.06325 | 0.071 | 0.065 | 0.067 | 0.06575 | 0.066 | 0.064 | 0.069 | 0.0655 | 0.066 | 0.06525 | 0.065 | 0.065 |
| 10 | 0.069 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.065 | | 0.066 | |
| 10 | 0.067 | | 0.063 | | 0.063 | | 0.063 | | 0.07 | | 0.064 | | 0.065 | | 0.064 | | 0.064 | |
| 10 | 0.081 | | 0.064 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.065 | | 0.066 | | 0.065 | |
| 11 | 0.082 | 0.07575 | 0.071 | 0.0655 | 0.064 | 0.0635 | 0.077 | 0.06675 | 0.066 | 0.06575 | 0.066 | 0.06425 | 0.069 | 0.0655 | 0.066 | 0.06525 | 0.065 | 0.065 |
| 11 | 0.07 | | 0.063 | | 0.063 | | 0.063 | | 0.064 | | 0.063 | | 0.064 | | 0.065 | | 0.066 | |
| 11 | 0.068 | | 0.064 | | 0.064 | | 0.064 | | 0.07 | | 0.064 | | 0.064 | | 0.064 | | 0.064 | |
| 11 | 0.083 | | 0.064 | | 0.063 | | 0.063 | | 0.063 | | 0.064 | | 0.065 | | 0.066 | | 0.065 | |
| 12 | 0.086 | 0.0775 | 0.07 | 0.06475 | 0.064 | 0.0635 | 0.072 | 0.0655 | 0.066 | 0.0655 | 0.066 | 0.06375 | 0.069 | 0.06575 | 0.067 | 0.0655 | 0.065 | 0.065 |
| 12 | 0.071 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.062 | | 0.064 | | 0.064 | | 0.066 | |
| 12 | 0.069 | | 0.063 | | 0.064 | | 0.064 | | 0.07 | | 0.064 | | 0.065 | | 0.064 | | 0.064 | |
| 12 | 0.084 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.063 | | 0.065 | | 0.067 | | 0.065 | |
| 13 | 0.092 | 0.0805 | 0.071 | 0.06575 | 0.065 | 0.06425 | 0.076 | 0.0665 | 0.066 | 0.06575 | 0.066 | 0.06425 | 0.07 | 0.06625 | 0.068 | 0.067 | 0.065 | 0.0755 |
| 13 | 0.073 | | 0.063 | | 0.064 | | 0.063 | | 0.064 | | 0.063 | | 0.064 | | 0.065 | | 0.108 | |
| 13 | 0.071 | | 0.064 | | 0.064 | | 0.064 | | 0.07 | | 0.064 | | 0.065 | | 0.066 | | 0.064 | |
| 13 | 0.086 | | 0.065 | | 0.064 | | 0.063 | | 0.063 | | 0.064 | | 0.066 | | 0.069 | | 0.065 | |
| 14 | 0.091 | 0.08375 | 0.071 | 0.06625 | 0.065 | 0.0645 | 0.074 | 0.06625 | 0.065 | 0.06575 | 0.066 | 0.06425 | 0.071 | 0.06725 | 0.07 | 0.06825 | 0.065 | 0.065 |
| 14 | 0.078 | | 0.064 | | 0.064 | | 0.063 | | 0.065 | | 0.063 | | 0.065 | | 0.066 | | 0.066 | |
| 14 | 0.075 | | 0.065 | | 0.065 | | 0.064 | | 0.07 | | 0.063 | | 0.066 | | 0.067 | | 0.064 | |
| 14 | 0.091 | | 0.065 | | 0.064 | | 0.064 | | 0.063 | | 0.065 | | 0.067 | | 0.07 | | 0.065 | |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 0.103 | 0.0935 | 0.073 | 0.0675 | 0.067 | 0.0655 | 0.075 | 0.067 | 0.067 | 0.067 | 0.067 | 0.06525 | 0.074 | 0.06875 | 0.076 | 0.07125 | 0.065 | 0.06525 |
| 15 | 0.088 | | 0.065 | | 0.065 | | 0.064 | | 0.066 | | 0.064 | | 0.066 | | 0.068 | | 0.067 | |
| 15 | 0.083 | | 0.066 | | 0.066 | | 0.065 | | 0.071 | | 0.065 | | 0.067 | | 0.068 | | 0.064 | |
| 15 | 0.1 | | 0.066 | | 0.064 | | 0.064 | | 0.064 | | 0.065 | | 0.068 | | 0.073 | | 0.065 | |
| 16 | 0.136 | 0.11675 | 0.076 | 0.06925 | 0.07 | 0.06675 | 0.079 | 0.0685 | 0.066 | 0.06725 | 0.068 | 0.066 | 0.081 | 0.0725 | 0.086 | 0.076 | 0.065 | 0.0655 |
| 16 | 0.108 | | 0.067 | | 0.065 | | 0.065 | | 0.067 | | 0.065 | | 0.069 | | 0.072 | | 0.067 | |
| 16 | 0.106 | | 0.067 | | 0.067 | | 0.065 | | 0.071 | | 0.066 | | 0.069 | | 0.07 | | 0.065 | |
| 16 | 0.117 | | 0.067 | | 0.065 | | 0.065 | | 0.065 | | 0.065 | | 0.071 | | 0.076 | | 0.065 | |
| 17 | 0.174 | 0.1465 | 0.084 | 0.0735 | 0.077 | 0.06975 | 0.081 | 0.07 | 0.067 | 0.06825 | 0.07 | 0.06725 | 0.096 | 0.0785 | 0.11 | 0.0875 | 0.066 | 0.066 |
| 17 | 0.13 | | 0.07 | | 0.066 | | 0.067 | | 0.068 | | 0.066 | | 0.074 | | 0.078 | | 0.068 | |
| 17 | 0.132 | | 0.07 | | 0.07 | | 0.066 | | 0.071 | | 0.066 | | 0.07 | | 0.075 | | 0.065 | |
| 17 | 0.15 | | 0.07 | | 0.066 | | 0.066 | | 0.067 | | 0.067 | | 0.074 | | 0.087 | | 0.065 | |
| 18 | 0.193 | 0.17775 | 0.104 | 0.08225 | 0.097 | 0.07725 | 0.088 | 0.073 | 0.068 | 0.06925 | 0.075 | 0.07 | 0.125 | 0.09175 | 0.138 | 0.10525 | 0.067 | 0.06675 |
| 18 | 0.158 | | 0.076 | | 0.07 | | 0.068 | | 0.07 | | 0.067 | | 0.08 | | 0.091 | | 0.068 | |
| 18 | 0.161 | | 0.075 | | 0.074 | | 0.067 | | 0.071 | | 0.068 | | 0.076 | | 0.087 | | 0.066 | |
| 18 | 0.199 | | 0.074 | | 0.068 | | 0.069 | | 0.068 | | 0.07 | | 0.086 | | 0.105 | | 0.066 | |
| 19 | 0.207 | 0.199 | 0.118 | 0.098 | 0.108 | 0.087 | 0.105 | 0.0805 | 0.07 | 0.07275 | 0.09 | 0.07675 | 0.158 | 0.1265 | 0.158 | 0.13925 | 0.07 | 0.06875 |
| 19 | 0.184 | | 0.098 | | 0.08 | | 0.073 | | 0.076 | | 0.072 | | 0.141 | | 0.135 | | 0.07 | |
| 19 | 0.196 | | 0.09 | | 0.085 | | 0.072 | | 0.073 | | 0.071 | | 0.1 | | 0.13 | | 0.067 | |
| 19 | 0.209 | | 0.086 | | 0.075 | | 0.072 | | 0.072 | | 0.074 | | 0.107 | | 0.134 | | 0.068 | |
| 20 | 0.213 | 0.221 | 0.142 | 0.12575 | 0.131 | 0.10925 | 0.129 | 0.09725 | 0.075 | 0.07725 | 0.11 | 0.0925 | 0.158 | 0.14725 | 0.17 | 0.15575 | 0.076 | 0.07125 |
| 20 | 0.207 | | 0.132 | | 0.11 | | 0.089 | | 0.083 | | 0.085 | | 0.142 | | 0.142 | | 0.072 | |
| 20 | 0.221 | | 0.126 | | 0.107 | | 0.087 | | 0.074 | | 0.094 | | 0.139 | | 0.142 | | 0.068 | |
| 20 | 0.243 | | 0.103 | | 0.089 | | 0.084 | | 0.077 | | 0.081 | | 0.15 | | 0.169 | | 0.069 | |
| 21 | 0.24 | 0.24525 | 0.159 | 0.15375 | 0.148 | 0.1425 | 0.154 | 0.13075 | 0.09 | 0.08725 | 0.141 | 0.13025 | 0.184 | 0.166 | 0.187 | 0.16775 | 0.09 | 0.07675 |
| 21 | 0.232 | | 0.161 | | 0.143 | | 0.132 | | 0.097 | | 0.138 | | 0.152 | | 0.146 | | 0.075 | |
| 21 | 0.239 | | 0.161 | | 0.166 | | 0.133 | | 0.076 | | 0.141 | | 0.163 | | 0.147 | | 0.07 | |
| 21 | 0.27 | | 0.134 | | 0.113 | | 0.104 | | 0.086 | | 0.101 | | 0.165 | | 0.191 | | 0.072 | |
| 22 | 0.275 | 0.2785 | 0.175 | 0.1755 | 0.164 | 0.16775 | 0.181 | 0.16575 | 0.114 | 0.104 | 0.158 | 0.1565 | 0.228 | 0.19375 | 0.222 | 0.19975 | 0.127 | 0.09175 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 22 | 0.266 | | 0.161 | | 0.183 | | 0.16 | | 0.12 | | 0.147 | | 0.185 | | 0.178 | | 0.086 | |
| 22 | 0.271 | | 0.198 | | 0.175 | | 0.174 | | 0.081 | | 0.172 | | 0.18 | | 0.169 | | 0.075 | |
| 22 | 0.302 | | 0.168 | | 0.149 | | 0.148 | | 0.101 | | 0.149 | | 0.182 | | 0.23 | | 0.079 | |
| 23 | 0.279 | 0.3045 | 0.184 | 0.18575 | 0.181 | 0.18075 | 0.19 | 0.17825 | 0.14 | 0.14375 | 0.173 | 0.16725 | 0.248 | 0.21525 | 0.269 | 0.23575 | 0.144 | 0.10675 |
| 23 | 0.313 | | 0.179 | | 0.172 | | 0.175 | | 0.163 | | 0.157 | | 0.188 | | 0.217 | | 0.1 | |
| 23 | 0.308 | | 0.18 | | 0.195 | | 0.169 | | 0.121 | | 0.168 | | 0.2 | | 0.207 | | 0.088 | |
| 23 | 0.318 | | 0.2 | | 0.175 | | 0.179 | | 0.151 | | 0.171 | | 0.225 | | 0.25 | | 0.095 | |
| 24 | 0.299 | 0.33225 | 0.209 | 0.219 | 0.208 | 0.211 | 0.219 | 0.1965 | 0.152 | 0.166 | 0.199 | 0.19 | 0.25 | 0.2285 | 0.266 | 0.2465 | 0.205 | 0.1365 |
| 24 | 0.345 | | 0.222 | | 0.207 | | 0.186 | | 0.187 | | 0.187 | | 0.224 | | 0.234 | | 0.124 | |
| 24 | 0.337 | | 0.216 | | 0.211 | | 0.193 | | 0.153 | | 0.191 | | 0.203 | | 0.221 | | 0.105 | |
| 24 | 0.348 | | 0.229 | | 0.218 | | 0.188 | | 0.172 | | 0.183 | | 0.237 | | 0.265 | | 0.112 | |
| 25 | 0.328 | 0.3565 | 0.231 | 0.25175 | 0.228 | 0.21675 | 0.233 | 0.22325 | 0.169 | 0.191 | 0.227 | 0.2165 | 0.283 | 0.26025 | 0.288 | 0.27275 | 0.189 | 0.15825 |
| 25 | 0.372 | | 0.287 | | 0.195 | | 0.212 | | 0.231 | | 0.218 | | 0.25 | | 0.256 | | 0.167 | |
| 25 | 0.357 | | 0.234 | | 0.225 | | 0.218 | | 0.18 | | 0.201 | | 0.243 | | 0.262 | | 0.133 | |
| 25 | 0.369 | | 0.255 | | 0.219 | | 0.23 | | 0.184 | | 0.22 | | 0.265 | | 0.285 | | 0.144 | |
| 26 | 0.336 | 0.36925 | 0.259 | 0.26575 | 0.253 | 0.2405 | 0.262 | 0.24825 | 0.184 | 0.2025 | 0.238 | 0.238 | 0.303 | 0.284 | 0.31 | 0.2935 | 0.23 | 0.1955 |
| 26 | 0.37 | | 0.266 | | 0.219 | | 0.247 | | 0.235 | | 0.242 | | 0.276 | | 0.272 | | 0.207 | |
| 26 | 0.39 | | 0.263 | | 0.239 | | 0.233 | | 0.173 | | 0.235 | | 0.263 | | 0.29 | | 0.159 | |
| 26 | 0.381 | | 0.275 | | 0.251 | | 0.251 | | 0.218 | | 0.237 | | 0.294 | | 0.302 | | 0.186 | |
| 27 | 0.357 | 0.37275 | 0.291 | 0.28125 | 0.28 | 0.26925 | 0.289 | 0.26825 | 0.216 | 0.233 | 0.253 | 0.26275 | 0.346 | 0.312 | 0.325 | 0.303 | 0.232 | 0.2205 |
| 27 | 0.387 | | 0.259 | | 0.246 | | 0.258 | | 0.275 | | 0.274 | | 0.3 | | 0.286 | | 0.244 | |
| 27 | 0.364 | | 0.268 | | 0.264 | | 0.258 | | 0.206 | | 0.262 | | 0.287 | | 0.29 | | 0.2 | |
| 27 | 0.383 | | 0.307 | | 0.287 | | 0.268 | | 0.235 | | 0.262 | | 0.315 | | 0.311 | | 0.206 | |
| 28 | 0.372 | 0.3865 | 0.332 | 0.29725 | 0.306 | 0.29125 | 0.31 | 0.286 | 0.234 | 0.2555 | 0.282 | 0.2875 | 0.351 | 0.32425 | 0.333 | 0.31625 | 0.242 | 0.241 |
| 28 | 0.403 | | 0.269 | | 0.269 | | 0.263 | | 0.303 | | 0.299 | | 0.318 | | 0.304 | | 0.26 | |
| 28 | 0.37 | | 0.266 | | 0.287 | | 0.287 | | 0.227 | | 0.289 | | 0.308 | | 0.301 | | 0.235 | |
| 28 | 0.401 | | 0.322 | | 0.303 | | 0.284 | | 0.258 | | 0.28 | | 0.32 | | 0.327 | | 0.227 | |
| 29 | 0.378 | 0.3875 | 0.347 | 0.30675 | 0.337 | 0.307 | 0.329 | 0.304 | 0.259 | 0.27425 | 0.304 | 0.30875 | 0.355 | 0.33925 | 0.435 | 0.35775 | 0.281 | 0.25975 |
| 29 | 0.377 | | 0.266 | | 0.273 | | 0.285 | | 0.303 | | 0.318 | | 0.328 | | 0.33 | | 0.26 | |

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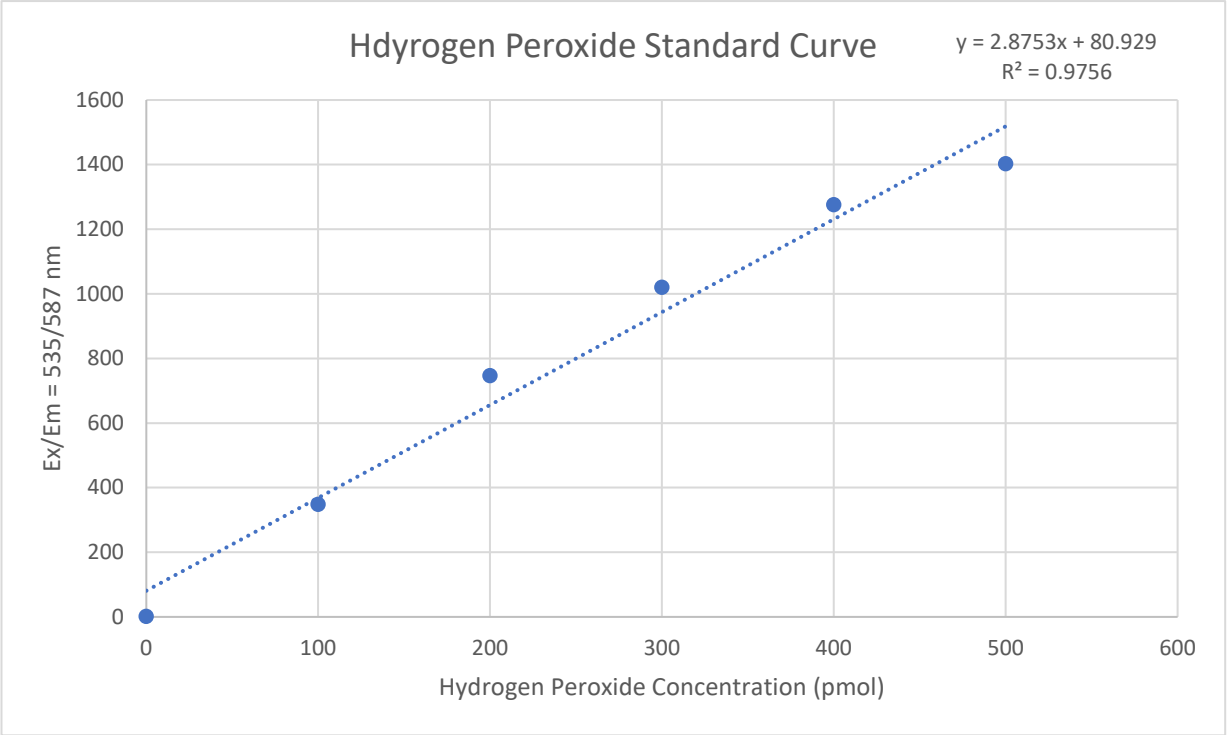
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 29 | 0.384 | | 0.281 | | 0.314 | | 0.304 | | 0.25 | | 0.302 | | 0.333 | | 0.325 | | 0.244 | |
| 29 | 0.411 | | 0.333 | | 0.304 | | 0.298 | | 0.285 | | 0.311 | | 0.341 | | 0.341 | | 0.254 | |
| 30 | 0.393 | 0.411 | 0.373 | 0.32325 | 0.407 | 0.33875 | 0.353 | 0.33125 | 0.273 | 0.30325 | 0.33 | 0.33325 | 0.437 | 0.37125 | 0.426 | 0.38175 | 0.296 | 0.2775 |
| 30 | 0.435 | | 0.282 | | 0.289 | | 0.336 | | 0.346 | | 0.341 | | 0.342 | | 0.395 | | 0.278 | |
| 30 | 0.412 | | 0.301 | | 0.342 | | 0.325 | | 0.274 | | 0.321 | | 0.346 | | 0.347 | | 0.257 | |
| 30 | 0.404 | | 0.337 | | 0.317 | | 0.311 | | 0.32 | | 0.341 | | 0.36 | | 0.359 | | 0.279 | |

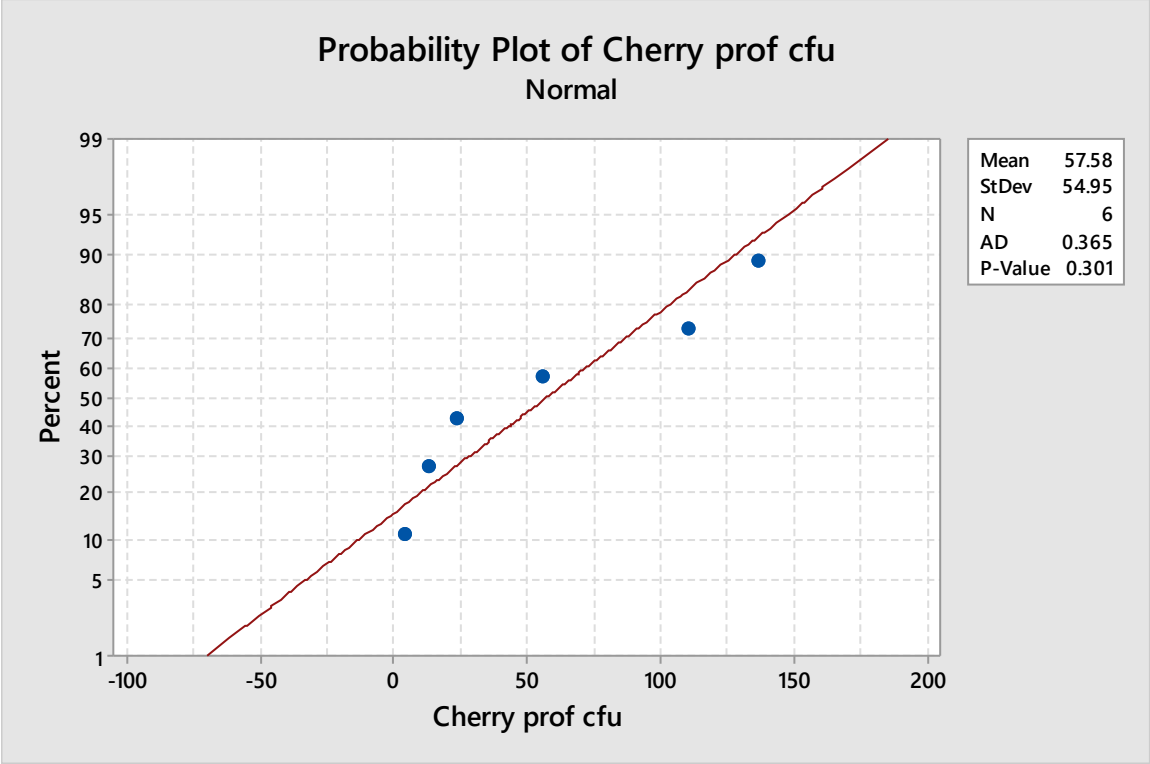
**Appendix 1.9 – Hydrogen peroxide standard used for quantitation**



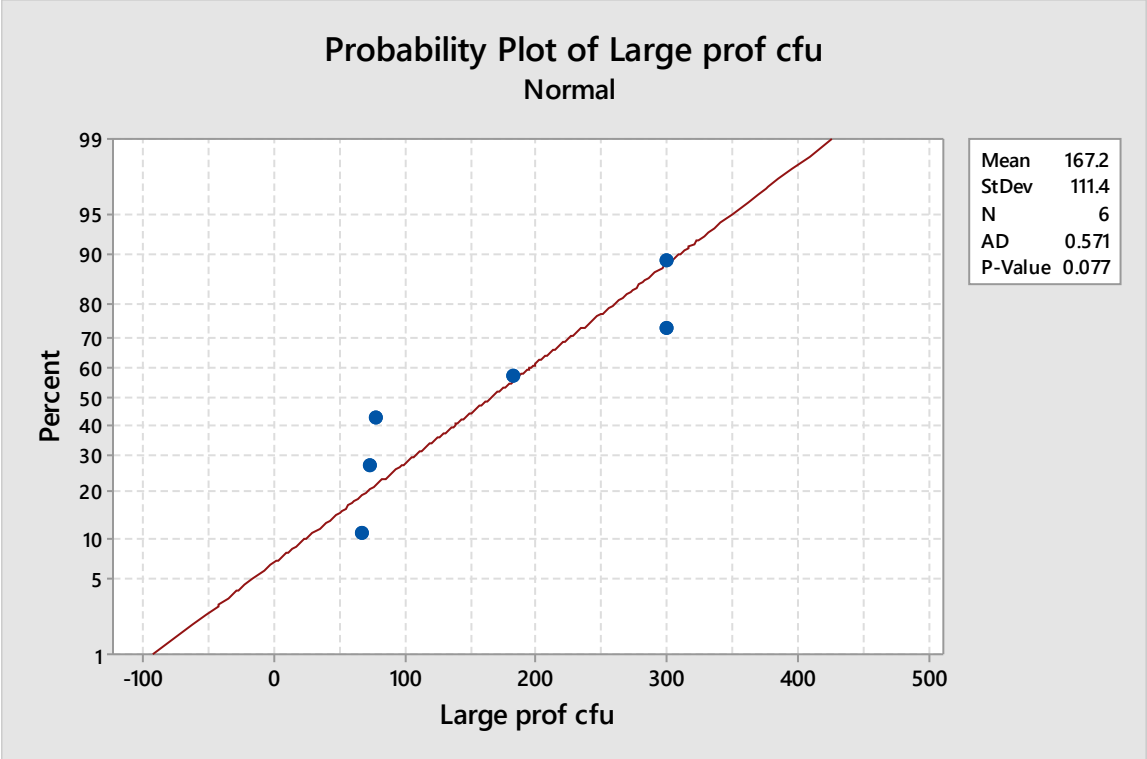
# Appendix 2 – Statistical Analysis

## Appendix 2.1 – *Salmonella* Proliferation

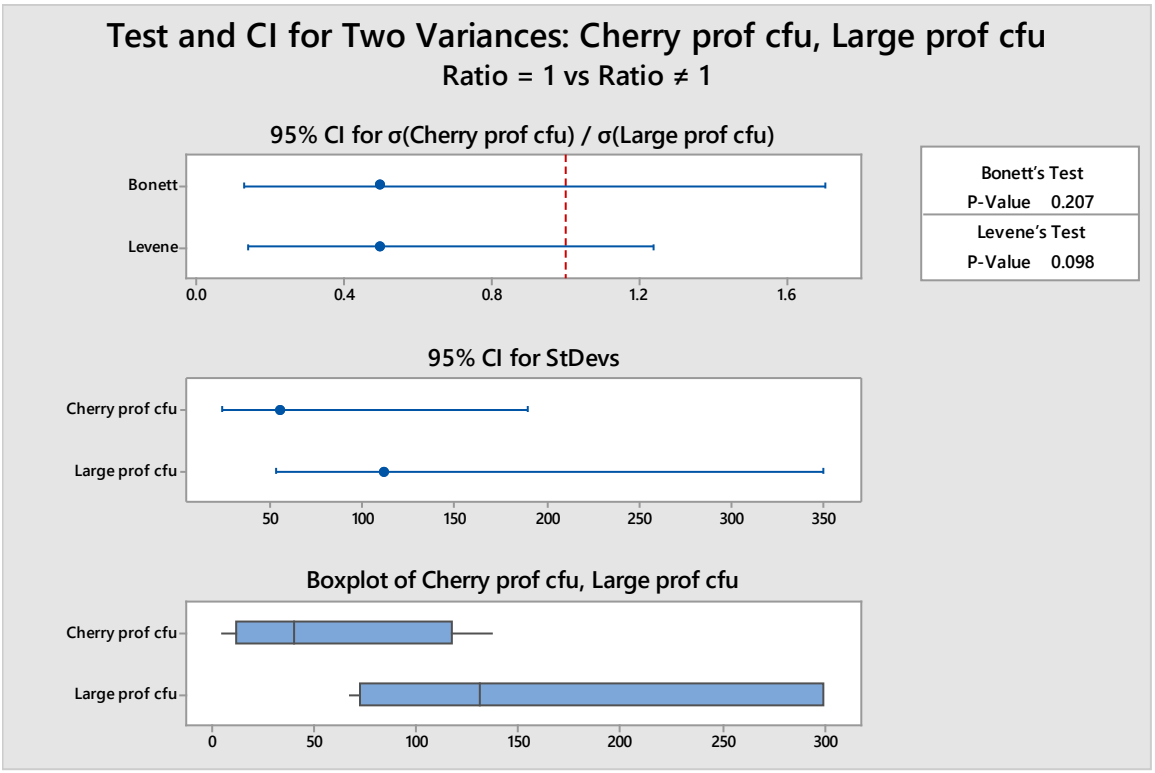
**Appendix 2.1.1** – Normality test for *Salmonella* proliferation in var. *cerasiforme* (CFU)



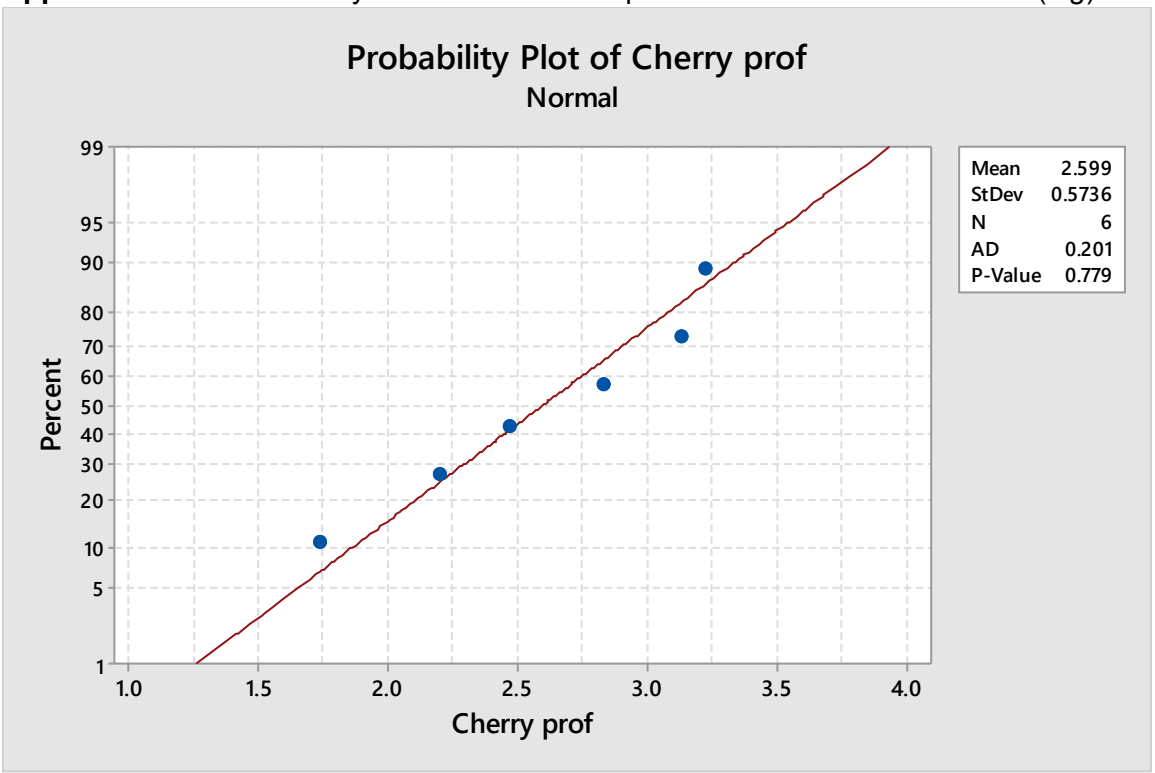

**Appendix 2.1.2** - Normality test for *Salmonella* proliferation in var. *alicante* (CFU)



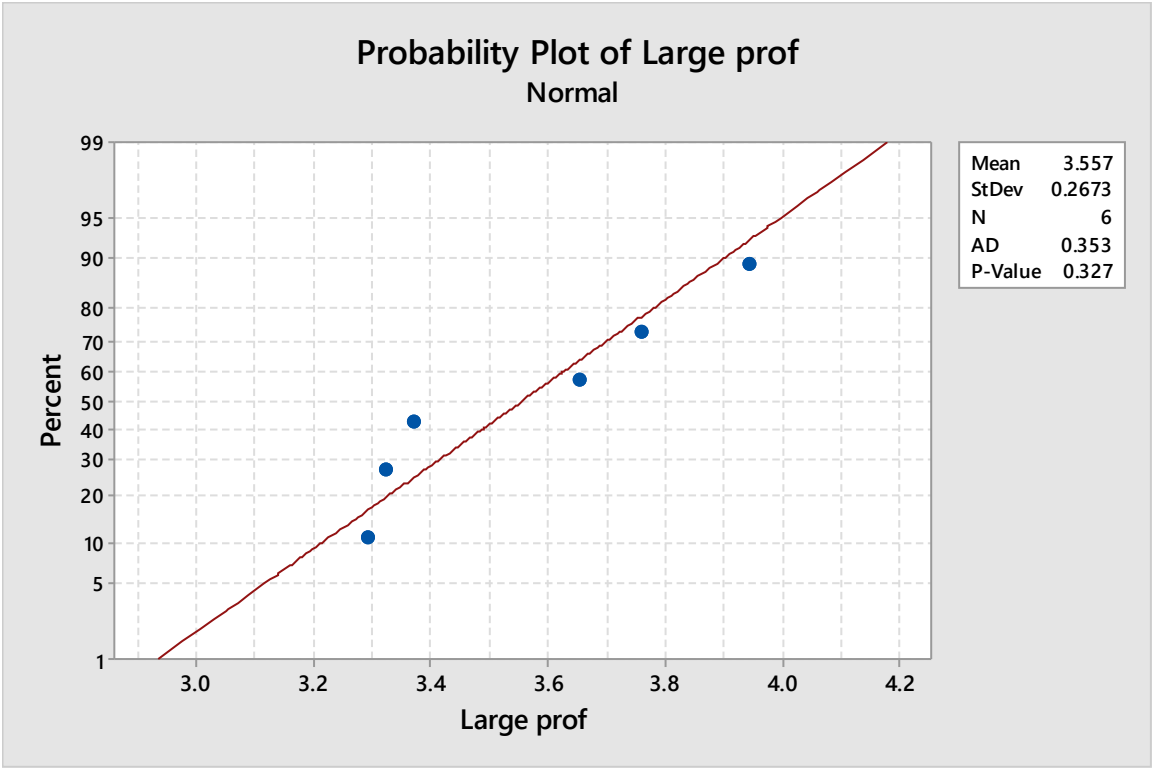
**Appendix 2.1.3** – Test for equal variances between *Salmonella* proliferation (CFU) in var. *cerasiforme* and var. *alicante*



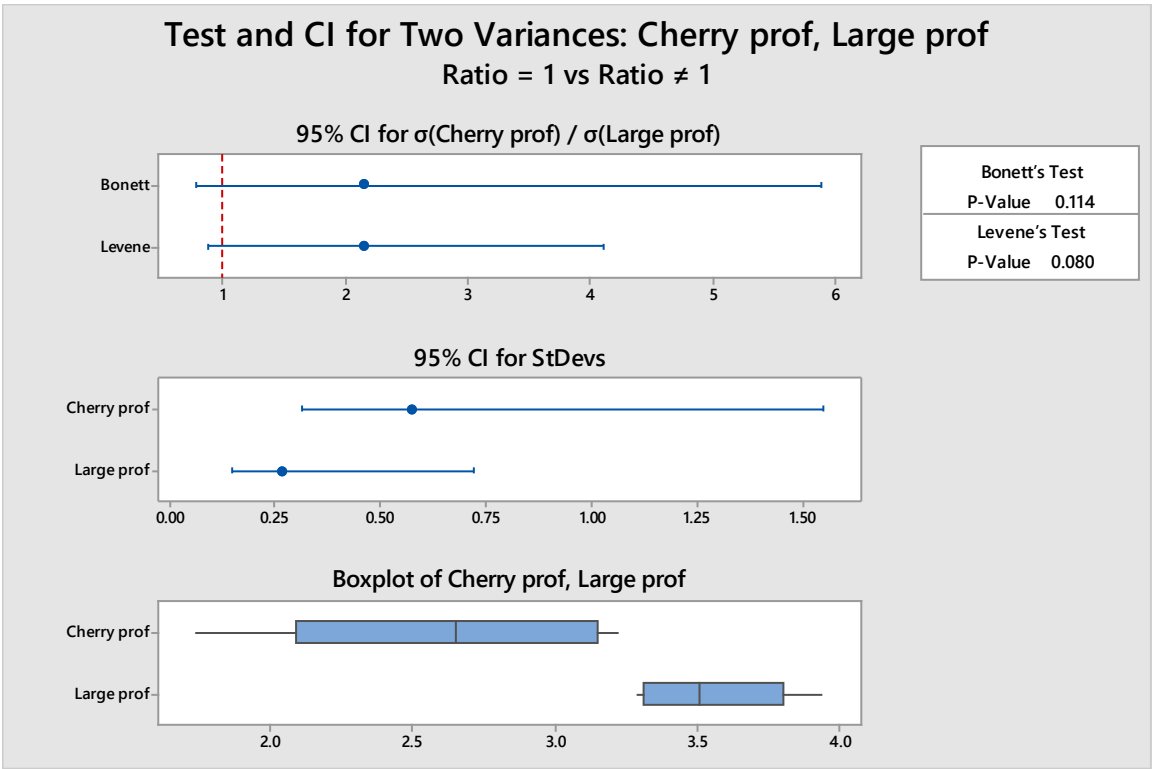

**Appendix 2.1.4 –** Normality test for *Salmonella* proliferation in var. *cerasiforme* (log)



**Appendix 2.1.5 –** Normality test for *Salmonella* proliferation in var. *alicante* (log)




**Appendix 2.1.6** – Test for equal variances between *Salmonella* proliferation (log) in var. *cerasiforme* and var. *alicante*

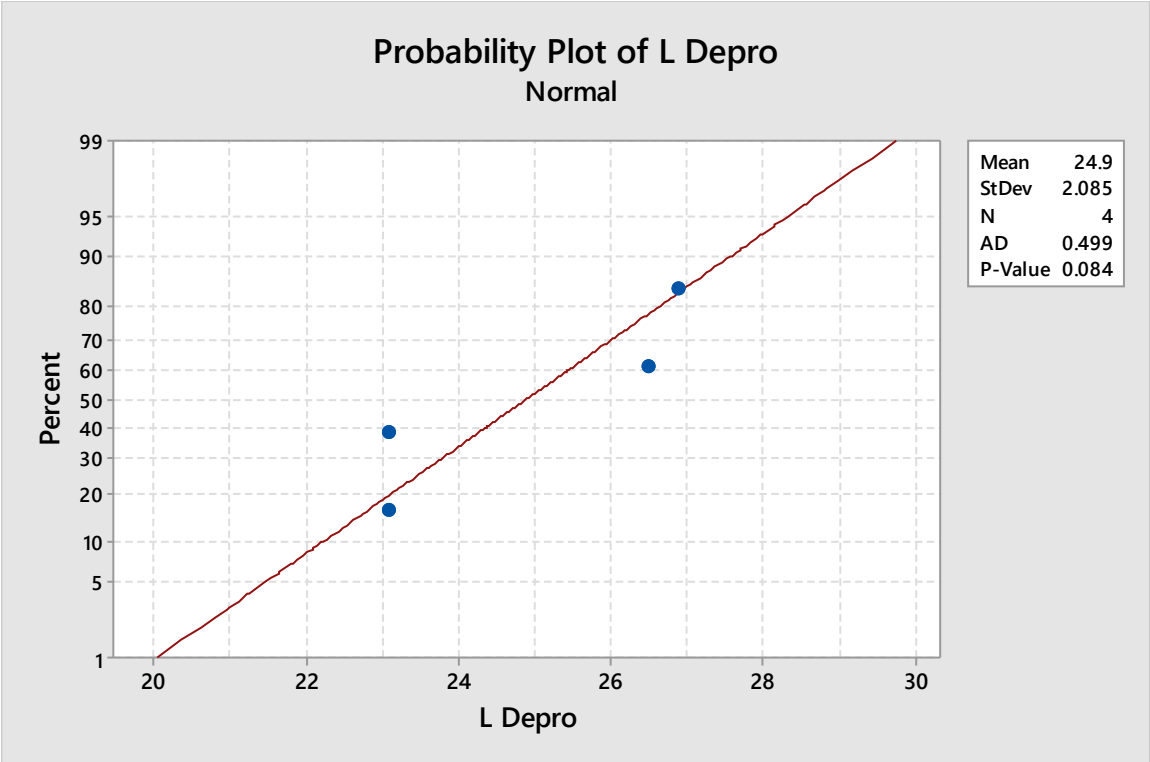


**Appendix 2.1.7 –** Two sample t-test of *Salmonella* proliferation between var. *cerasiforme* and var. *alicante*

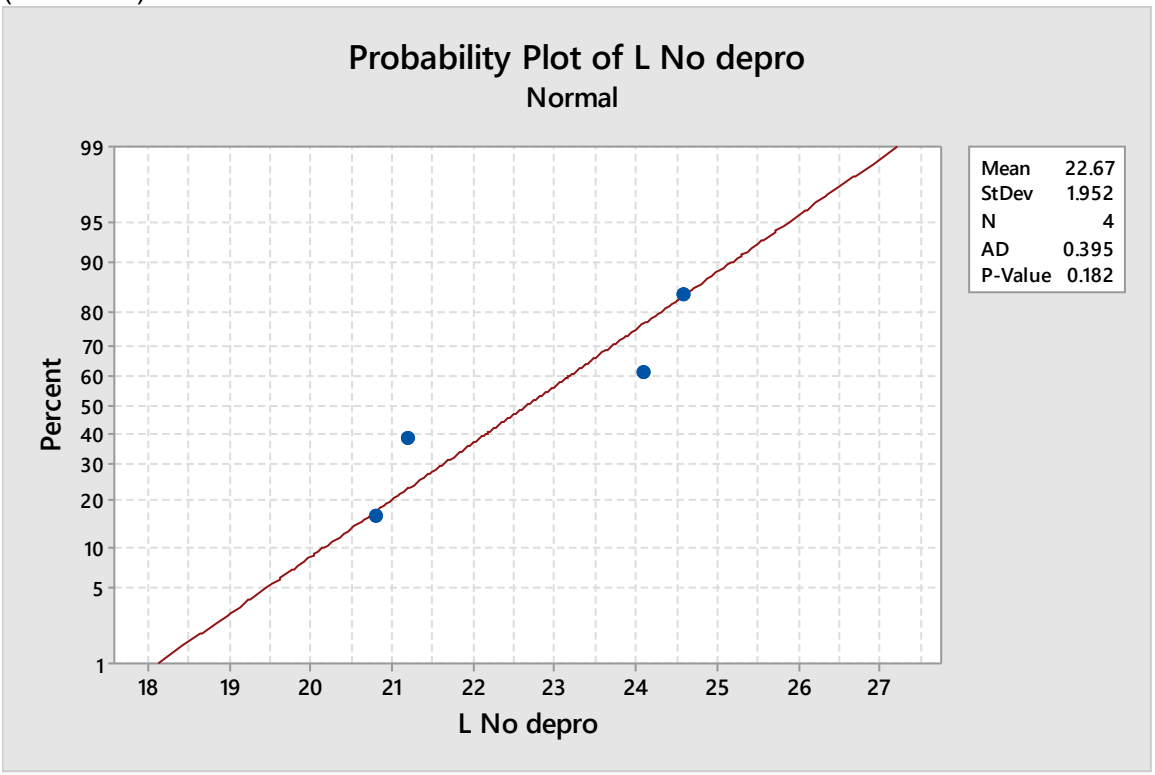
## Two-Sample T-Test and CI: Cherry prof, Large prof

```
Two-sample T for Cherry prof vs Large prof

             N   Mean  StDev  SE Mean
Cherry prof  6  2.599  0.574     0.23
Large prof   6  3.557  0.267     0.11


Difference = μ (Cherry prof) - μ (Large prof)
Estimate for difference:  -0.958
95% CI for difference:  (-1.534, -0.383)
T-Test of difference = 0 (vs ≠): T-Value = -3.71  P-Value = 0.004  DF = 10
Both use Pooled StDev = 0.4475
```

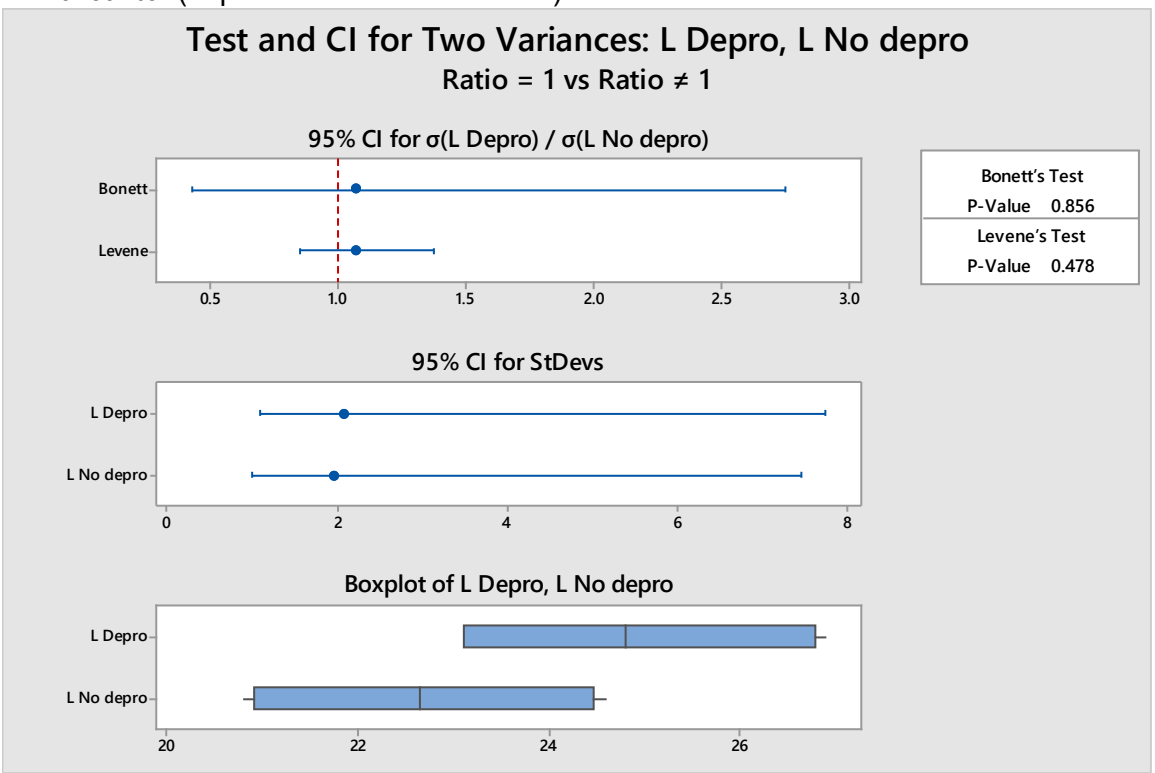
**Appendix 2.2.1 –** Normality test of hydrogen peroxide concentration in var. *alicante* (deproteinised)



**Appendix 2.2.2 -** Normality test of hydrogen peroxide concentration in var. *alicante* (untreated)




**Appendix 2.2.3 –** Test for equal variance between hydrogen peroxide concentration in var. *alicante* (deproteinised and untreated)

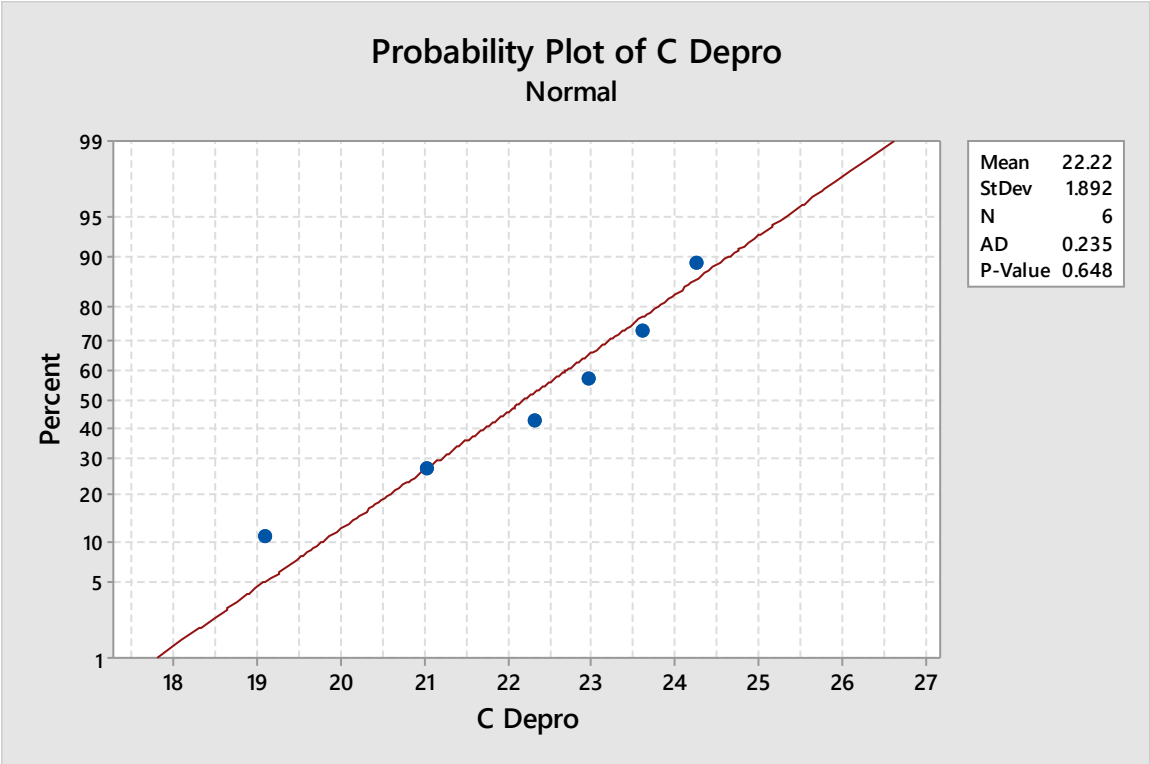


**Appendix 2.2.4** – Two sample t-test of hydrogen peroxide concentration in var. *alicante* (deproteinised and untreated)

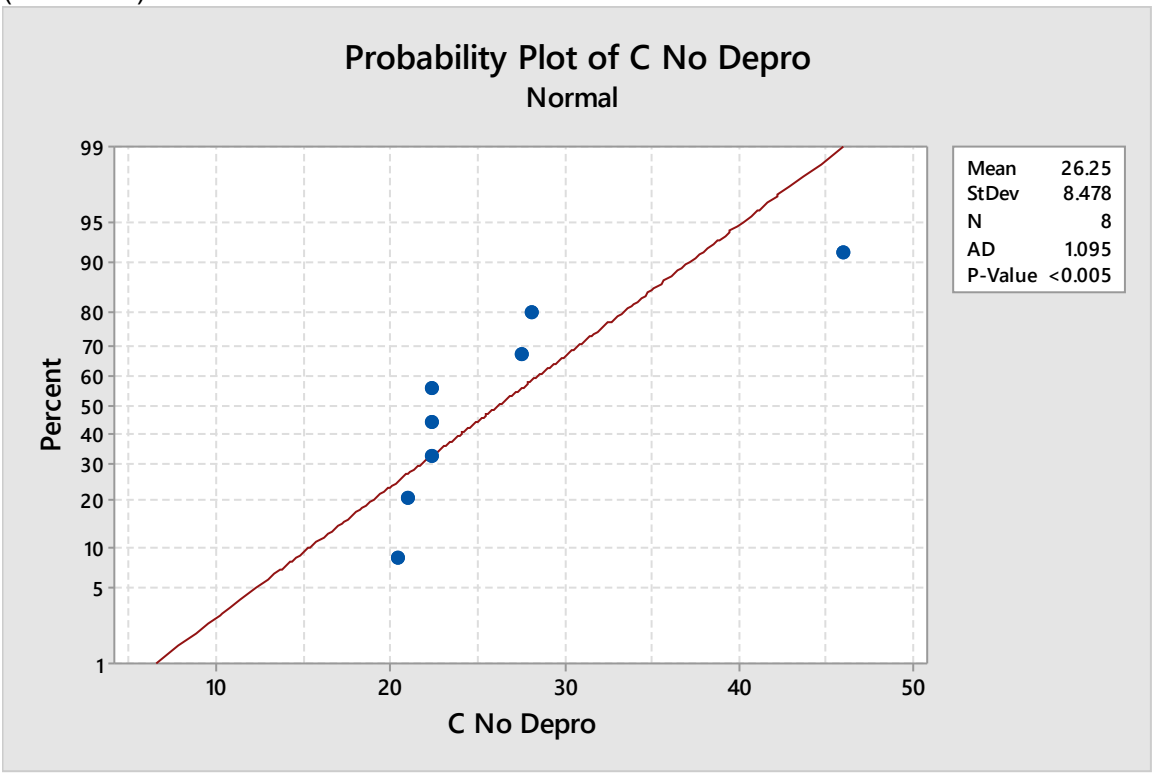
**Two-Sample T-Test and CI: L Depro, L No depro**

```
Two-sample T for L Depro vs L No depro

             N   Mean  StDev  SE Mean
L Depro      4  24.90   2.08      1.0
L No depro   4  22.67   1.95     0.98


Difference = μ (L Depro) - μ (L No depro)
Estimate for difference:  2.23
95% CI for difference:  (-1.27, 5.72)
T-Test of difference = 0 (vs ≠): T-Value = 1.56  P-Value = 0.170  DF = 6
Both use Pooled StDev = 2.0194
```

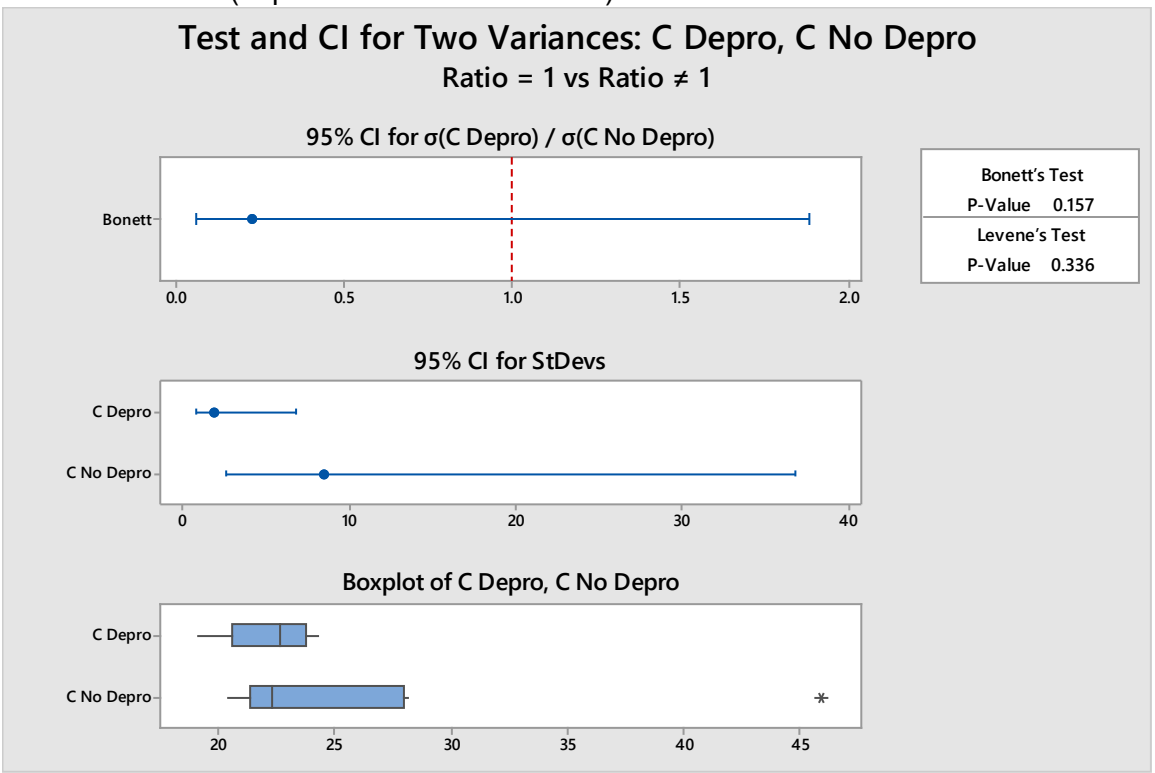
**Appendix 2.2.5 -** Normality test of hydrogen peroxide concentration in var. *cerasiforme* (deproteinised)



**Appendix 2.2.6 -** Normality test of hydrogen peroxide concentration in var. *cerasiforme* (untreated)




**Appendix 2.2.7 –** Test for equal variance between hydrogen peroxide concentration in var. *cerasiforme* (deproteinised and untreated)

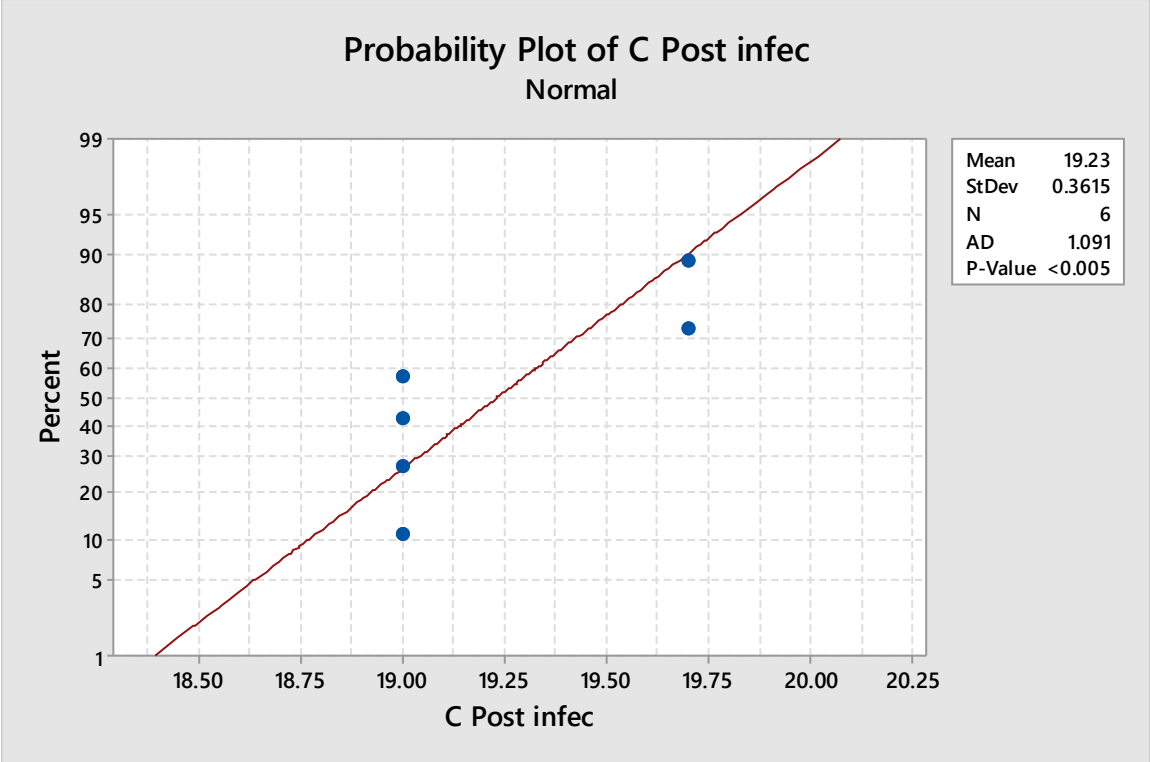


**Appendix 2.2.8** – Two sample t-test of hydrogen peroxide concentration in var. *cerasiforme* (deproteinised and untreated)

**Two-Sample T-Test and CI: C Depro, C No Depro**

```
Two-sample T for C Depro vs C No Depro

             N   Mean  StDev  SE Mean
C Depro      6  22.22   1.89     0.77
C No Depro   8  26.25   8.48      3.0


Difference = μ (C Depro) - μ (C No Depro)
Estimate for difference:  -4.04
95% CI for difference:  (-11.36, 3.28)
T-Test of difference = 0 (vs ≠): T-Value = -1.30  P-Value = 0.233  DF = 7
```

**Appendix 2.3.1 –** Normality test for hydrogen peroxide concentration in var. *cerasiforme* post infection from *Salmonella*

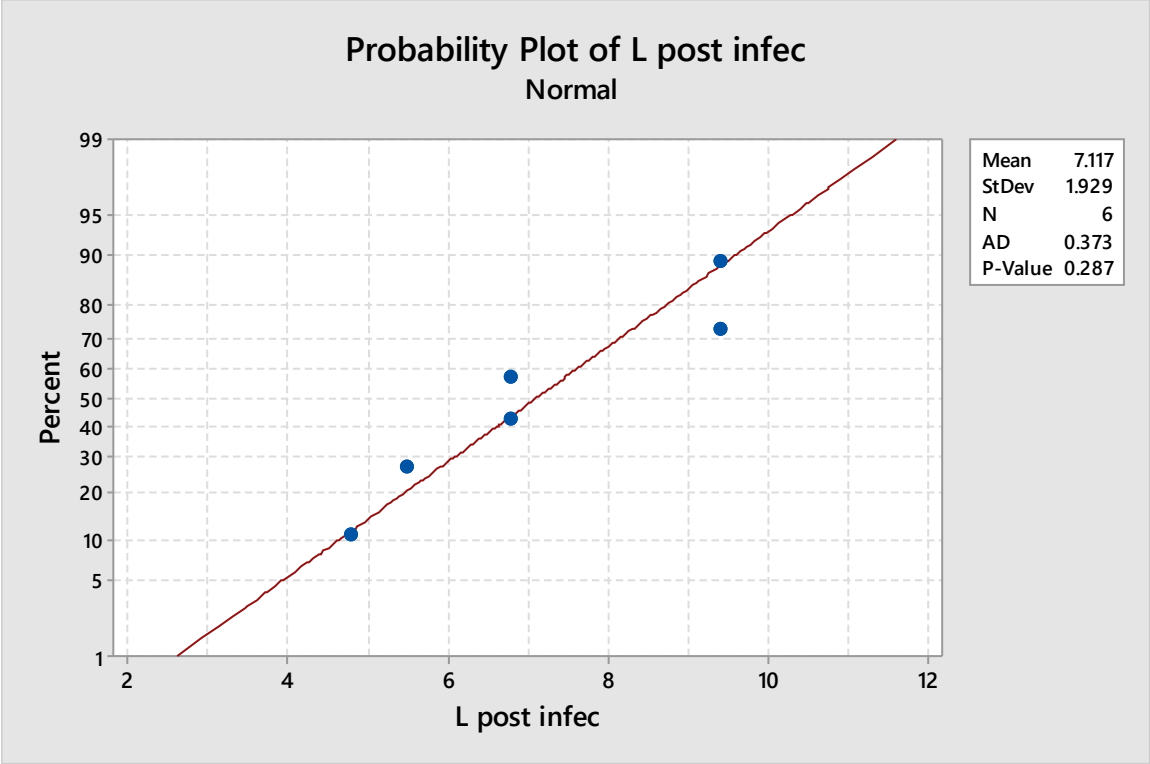



**Appendix 2.3.2** – Man-Whitney test of hydrogen peroxide concentration in var. *cerasiforme* in post infection vs untreated.

**Mann-Whitney Test and CI: C Post infec, C Depro**

```
              N  Median
C Post infec  6  19.000
C Depro       6  22.645


Point estimate for η1 - η2 is -3.320
95.5 Percent CI for η1 - η2 is (-4.620,-0.091)
W = 23.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0131
The test is significant at 0.0114 (adjusted for ties)
```

**Appendix 2.3.3 –** Normality test for hydrogen peroxide concentration in var. *alicante* post infection from *Salmonella*

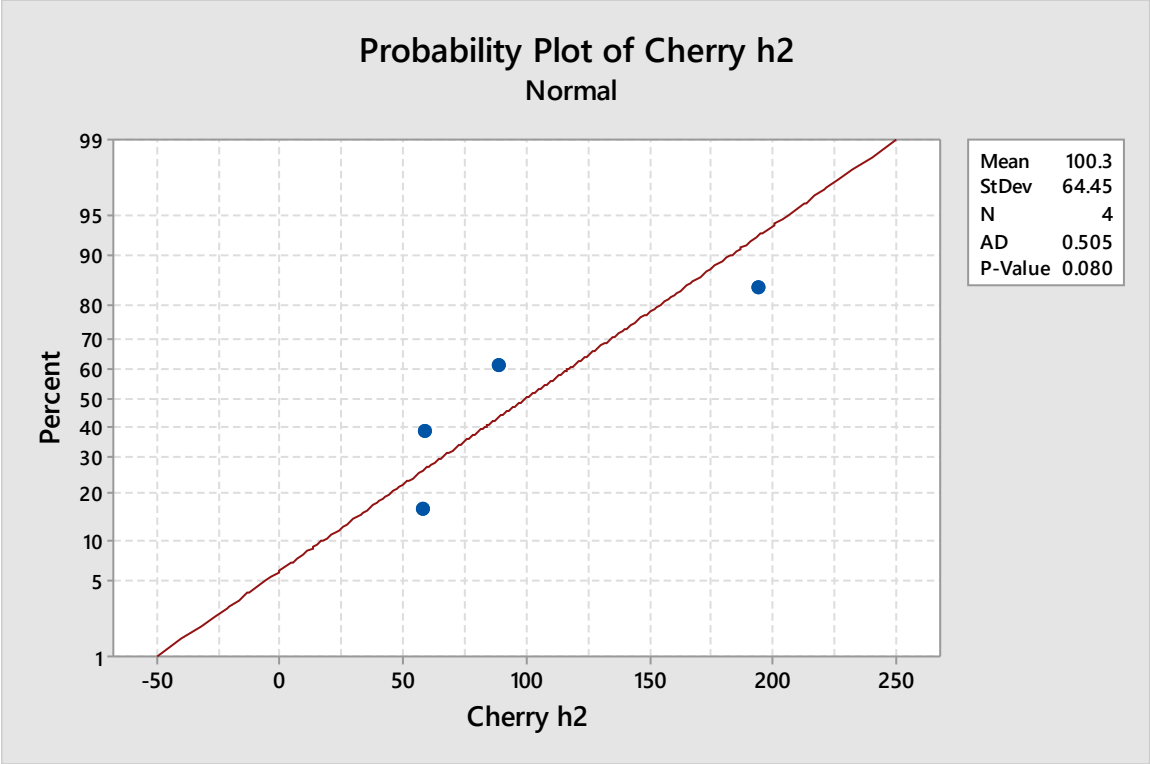



**Appendix 2.3.2** – Two samples t-test test of hydrogen peroxide concentration in var. *alicante* in post infection vs untreated.

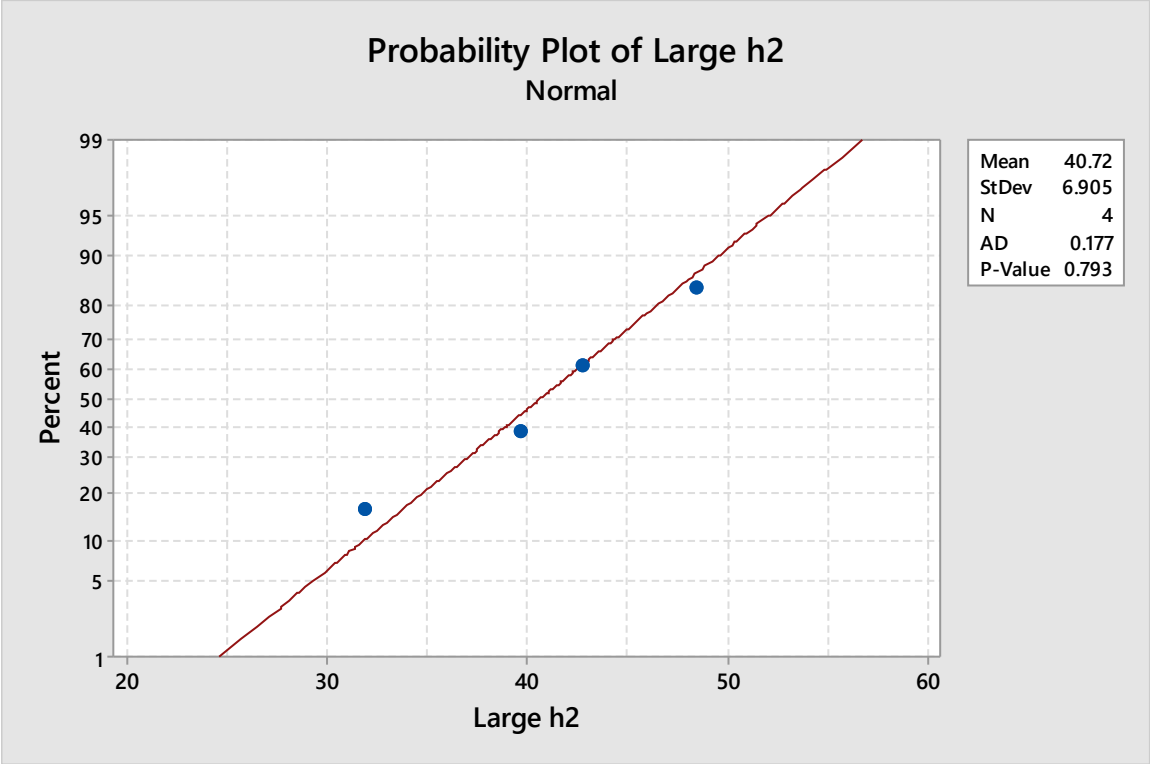
**Two-Sample T-Test and CI: L post infec, Large prof**

```
Two-sample T for L post infec vs Large prof

              N   Mean  StDev  SE Mean
L post infec  6   7.12   1.93     0.79
Large prof    6  3.557  0.267     0.11


Difference = μ (L post infec) - μ (Large prof)
Estimate for difference:  3.559
95% CI for difference:  (1.788, 5.331)
T-Test of difference = 0 (vs ≠): T-Value = 4.48  P-Value = 0.001  DF = 10
Both use Pooled StDev = 1.3772
```

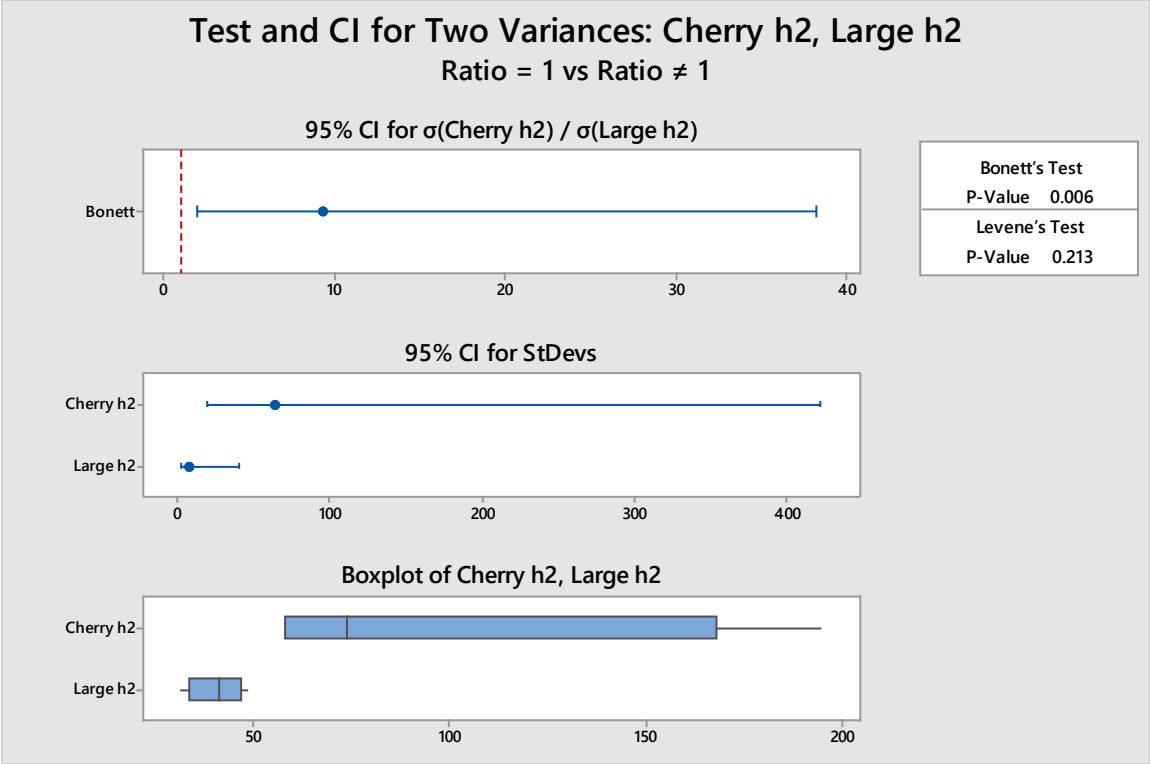
**Appendix 2.4.1 –** Normality for hydrogen peroxide concentration in var. *cerasiforme* (second run)




**Appendix 2.4.2 –** Normality for hydrogen peroxide concentration in var. *alicante* (second run)



**Appendix 2.4.3 –** Test for equal variances for hydrogen peroxide concentration in var. *alicante* and var. *cerasiforme* (second run)

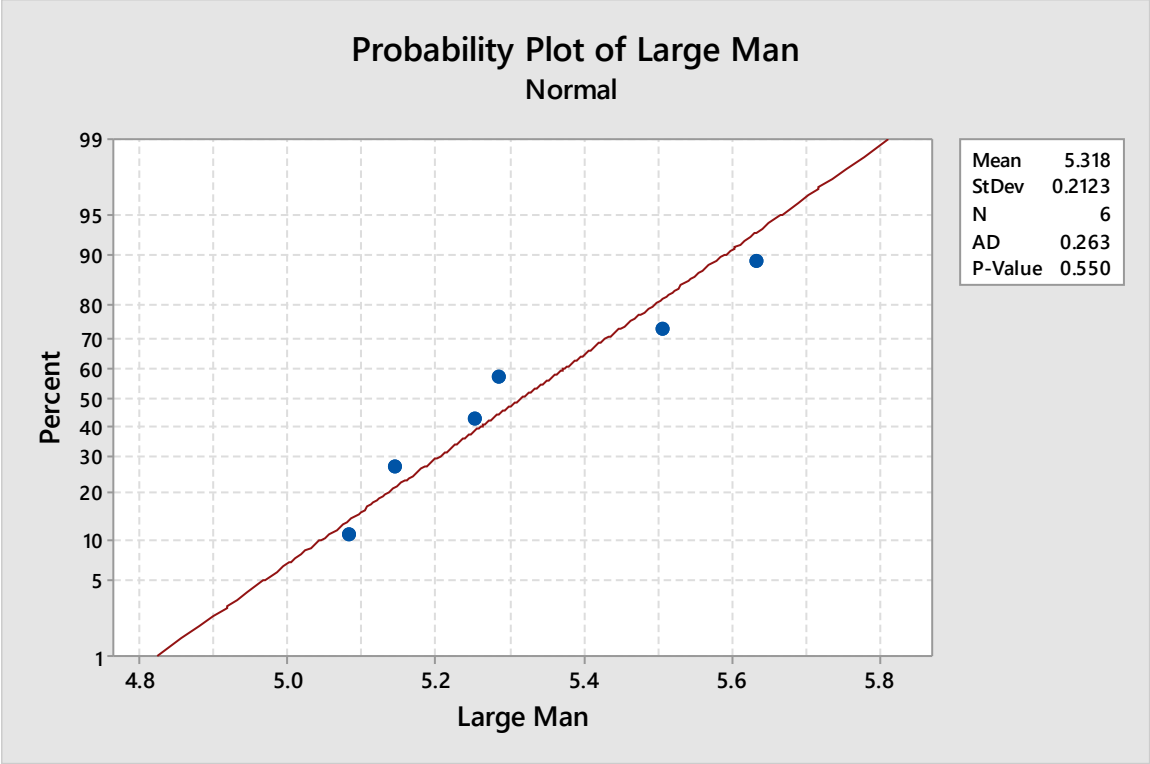



**Appendix 2.4.4 –** Man-Whitney test of hydrogen peroxide concentration in var. *cerasiforme* and var. *alicante* (second run).

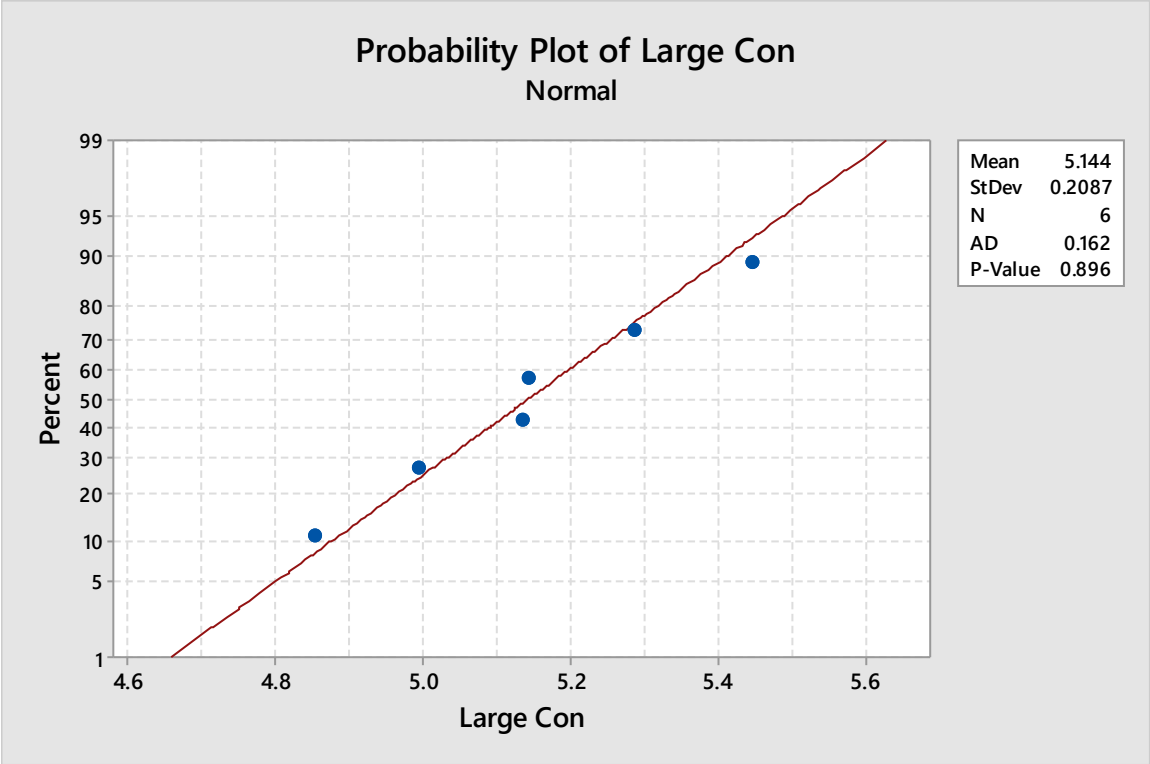
## Mann-Whitney Test and CI: Cherry h2, Large h2

```
           N  Median
Cherry h2  4    74.3
Large h2   4    41.3


Point estimate for η1 - η2 is 34.1
97.0 Percent CI for η1 - η2 is (9.7,162.7)
W = 26.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0304
```

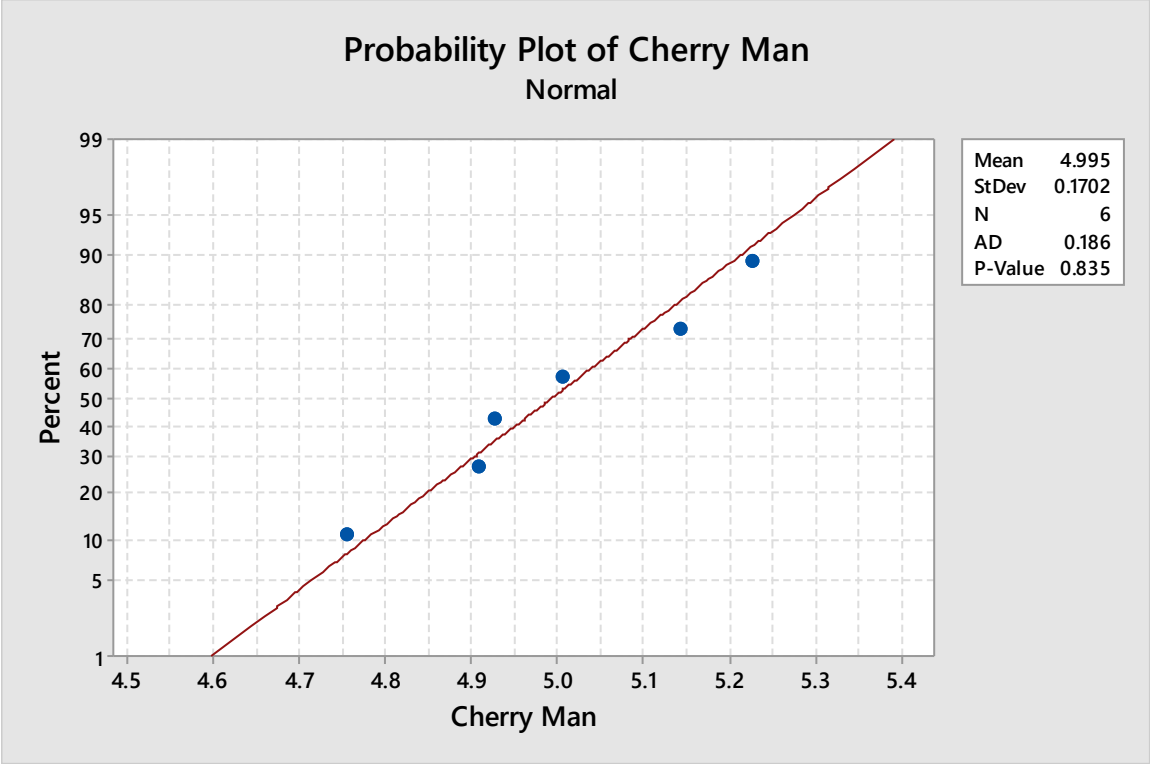
**Appendix 2.5.1 –** Normality test for *Salmonella* proliferation in var. *alicante* (mannitol)



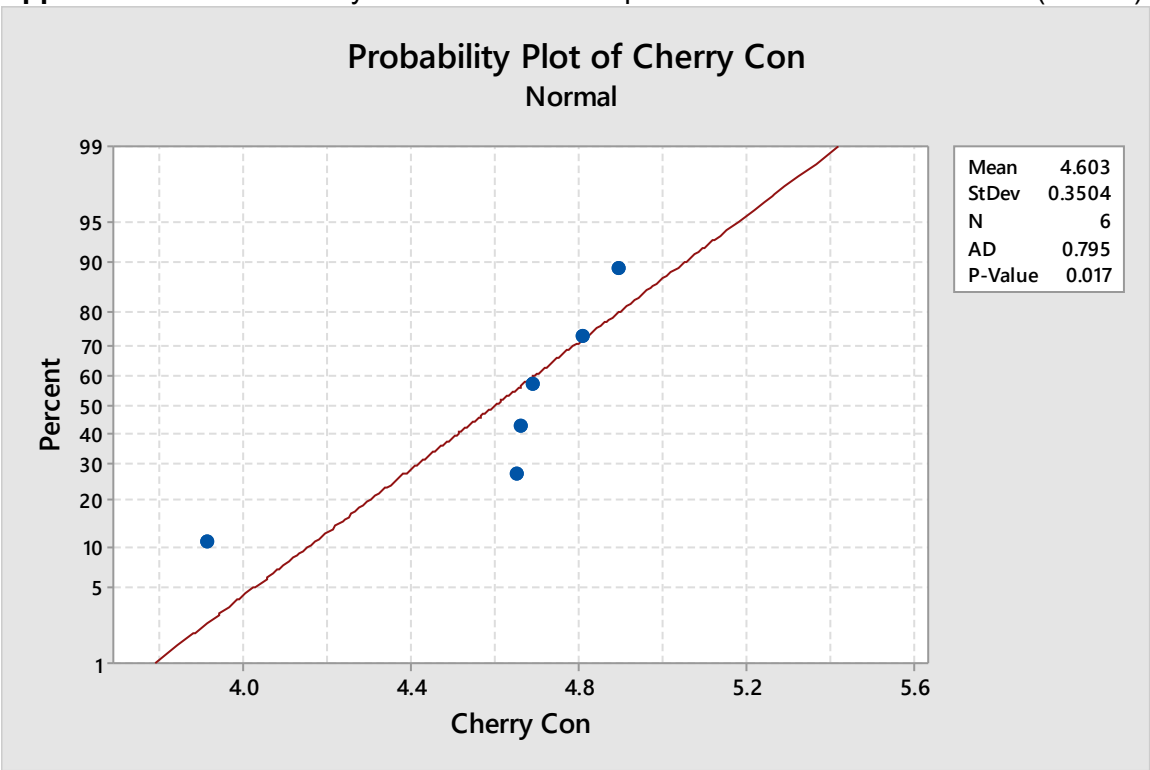

**Appendix 2.5.2 -** Normality test for *Salmonella* proliferation in var. *alicante* (control)



**Appendix 2.5.3 –** Normality test for *Salmonella* proliferation in var. *cerasiforme* (mannitol)




**Appendix 2.5.3 –** Normality test for *Salmonella* proliferation in var. *cerasiforme* (control)



**Appendix 2.5.3** – Two samples t-test test of *Salmonella* proliferation in var. *alicante* (control vs. mannitol)

## Two-Sample T-Test and CI: Large Man, Large Con

```
Two-sample T for Large Man vs Large Con

           N   Mean  StDev  SE Mean
Large Man  6  5.318  0.212    0.087
Large Con  6  5.144  0.209    0.085


Difference = μ (Large Man) - μ (Large Con)
Estimate for difference:  0.174
95% CI for difference:  (-0.097, 0.445)
T-Test of difference = 0 (vs ≠): T-Value = 1.43  P-Value = 0.182  DF = 10
Both use Pooled StDev = 0.2105
```

**Appendix 2.5.4 –** Man-Whitney test of *Salmonella* proliferation in var. *cerasiforme* (control vs. mannitol).

## Mann-Whitney Test and CI: Cherry Man, Cherry Con

```
            N  Median
Cherry Man  6  4.9670
Cherry Con  6  4.6747


Point estimate for η1 - η2 is 0.2953
95.5 Percent CI for η1 - η2 is (0.0968,0.8421)
W = 55.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0131
```

**Appendix 2.5.4 –** Man-Whitney test of *Salmonella* proliferation in var. *cerasiforme* vs. var. *alicante* (control)

## Mann-Whitney Test and CI: Cherry Con, Large Con

```
            N  Median
Cherry Con  6  4.6747
Large Con   6  5.1400


Point estimate for η1 - η2 is -0.4785
95.5 Percent CI for η1 - η2 is (-0.9408,-0.1954)
W = 22.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0082
```

**Appendix 2.5.6** – Two samples t-test test of *Salmonella* proliferation in var. *alicante* vs. var. *cerasiforme* (mannitol)

## Two-Sample T-Test and CI: Cherry Man, Large Man

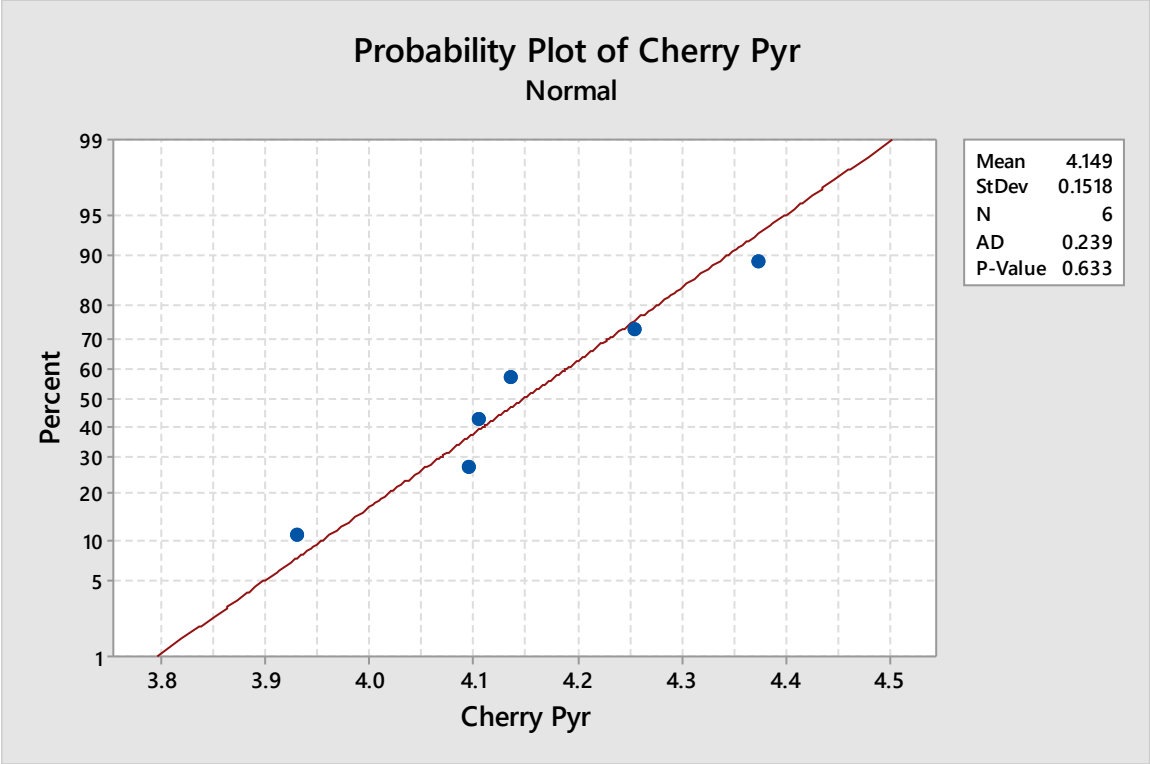
```
Two-sample T for Cherry Man vs Large Man

            N   Mean  StDev  SE Mean
Cherry Man  6  4.995  0.170    0.069
Large Man   6  5.318  0.212    0.087


Difference = μ (Cherry Man) - μ (Large Man)
Estimate for difference:  -0.323
95% CI for difference:  (-0.570, -0.075)
T-Test of difference = 0 (vs ≠): T-Value = -2.91  P-Value = 0.016  DF = 10
Both use Pooled StDev = 0.1924
```

**Appendix 2.5.6** – Summarised Two samples t-test test of estimated difference in *Salmonella* proliferation in var. *alicante* vs. var. *cerasiforme* (mannitol) and var. *alicante* vs. var. *cerasiforme* (control)

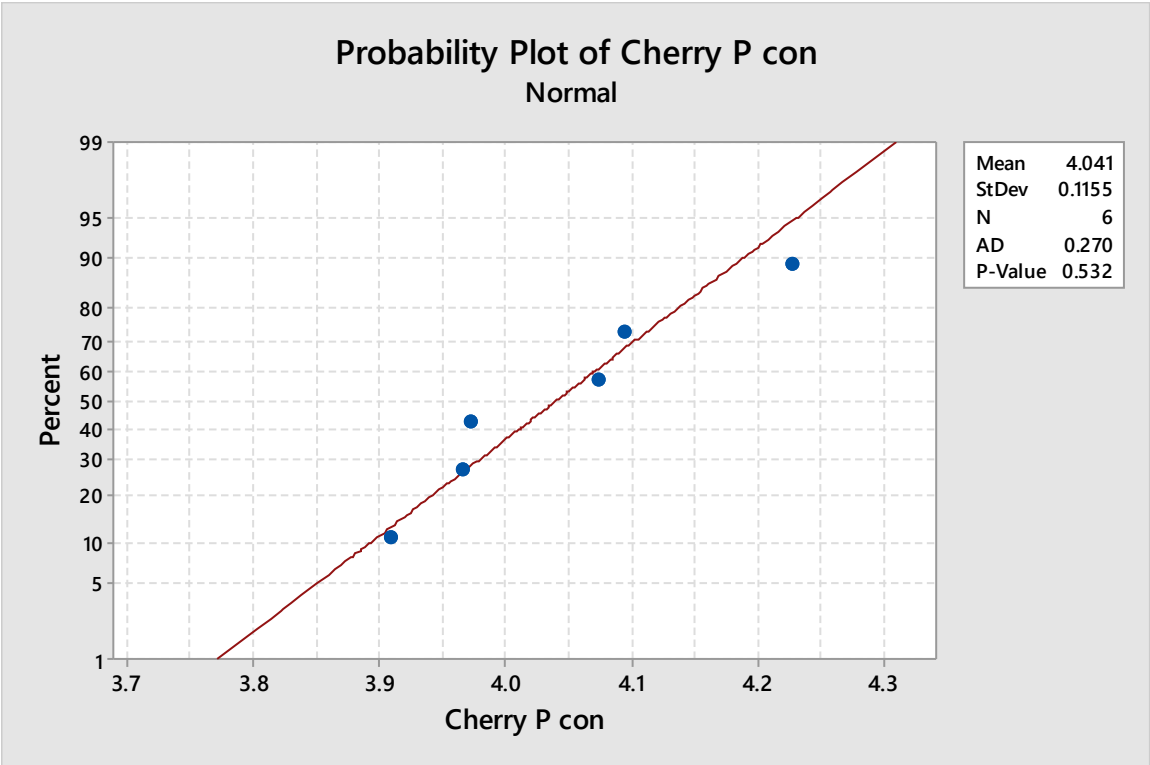
## Two-Sample T-Test and CI

```
Sample  N   Mean  StDev  SE Mean
1       6  0.323  0.176    0.072
2       6  0.541  0.176    0.072


Difference = μ (1) - μ (2)
Estimate for difference:  -0.218
95% CI for difference:  (-0.444, 0.008)
T-Test of difference = 0 (vs ≠): T-Value = -2.15  P-Value = 0.057  DF = 10
Both use Pooled StDev = 0.1756
```

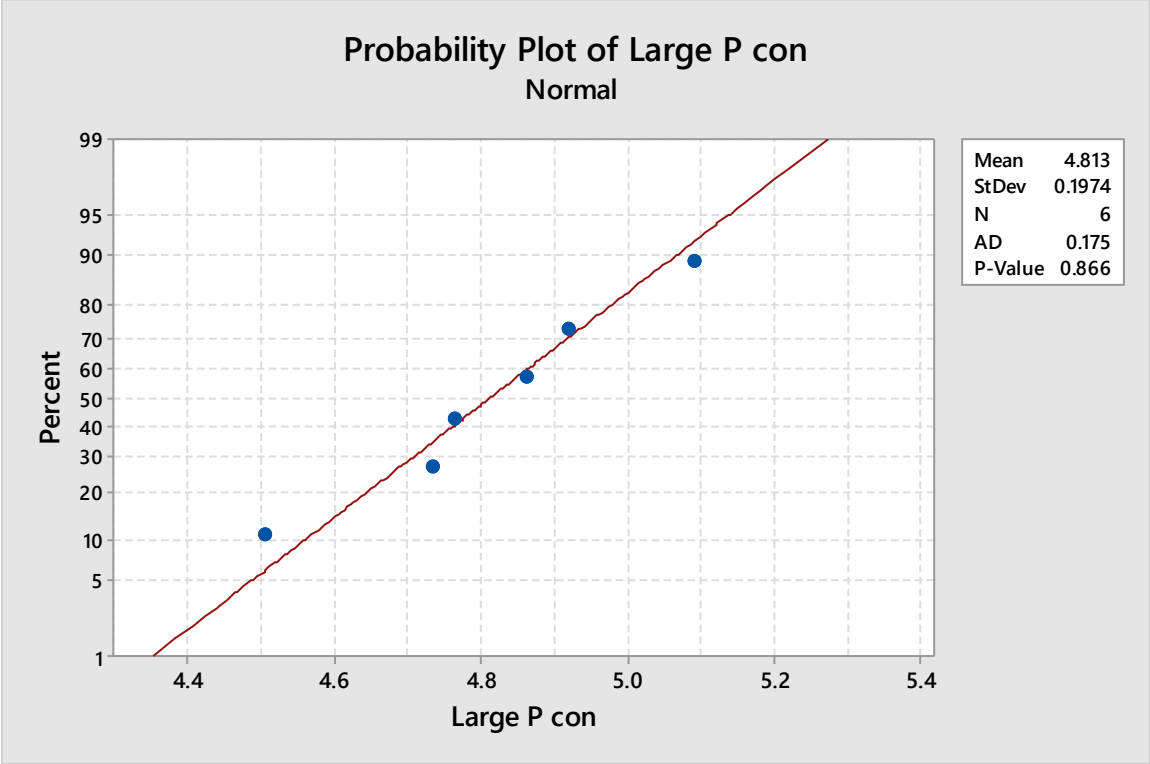
**Appendix 2.6.1** - Normality test for *Salmonella* proliferation in var. *cerasiforme* (sodium pyruvate)



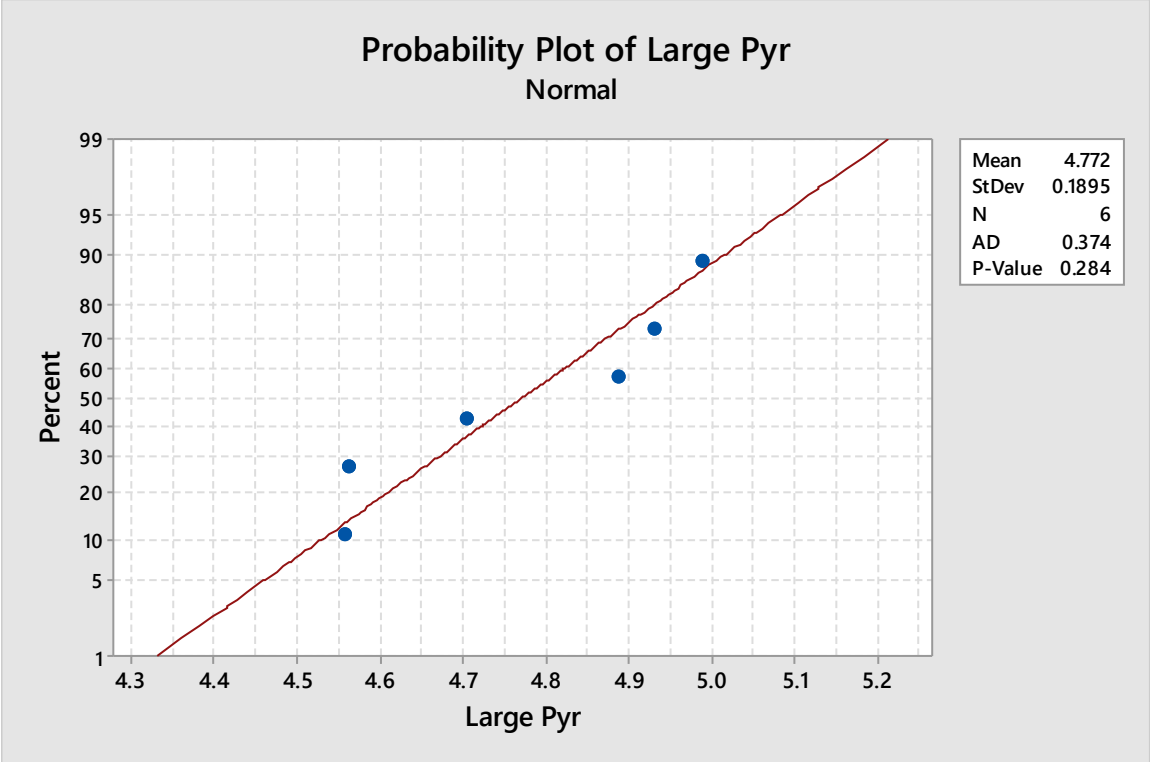

**Appendix 2.6.2** - Normality test for *Salmonella* proliferation in var. *cerasiforme* (control)



**Appendix 2.6.3** - Normality test for *Salmonella* proliferation in var. *alicante* (control)




**Appendix 2.6.3** - Normality test for *Salmonella* proliferation in var. *alicante* (sodium pyruvate)



**Appendix 2.6.4** – Two samples t-test test of *Salmonella* proliferation in var. *alicante* vs. var. *cerasiforme* (control)

## Two-Sample T-Test and CI: Cherry P con, Large P con

```
Two-sample T for Cherry P con vs Large P con

              N   Mean  StDev  SE Mean
Cherry P con  6  4.041  0.115    0.047
Large P con   6  4.813  0.197    0.081


Difference = μ (Cherry P con) - μ (Large P con)
Estimate for difference:  -0.7726
95% CI for difference:  (-0.9806, -0.5646)
T-Test of difference = 0 (vs ≠): T-Value = -8.27  P-Value = 0.000  DF = 10
Both use Pooled StDev = 0.1617
```

**Appendix 2.6.4** – Two samples t-test test of *Salmonella* proliferation in var. *alicante* vs. var. *cerasiforme* (sodium pyruvate)

## Two-Sample T-Test and CI: Cherry Pyr, Large Pyr

```
Two-sample T for Cherry Pyr vs Large Pyr

            N   Mean  StDev  SE Mean
Cherry Pyr  6  4.149  0.152    0.062
Large Pyr   6  4.772  0.189    0.077


Difference = μ (Cherry Pyr) - μ (Large Pyr)
Estimate for difference:  -0.6229
95% CI for difference:  (-0.8437, -0.4020)
T-Test of difference = 0 (vs ≠): T-Value = -6.28  P-Value = 0.000  DF = 10
Both use Pooled StDev = 0.1717
```

**Appendix 2.5.6** – Summarised Two samples t-test test of estimated difference in *Salmonella* proliferation in var. *alicante* vs. var. *cerasiforme* (sodium pyruvate) and var. *alicante* vs. var. *cerasiforme* (control)

## Two-Sample T-Test and CI

```
Sample  N   Mean  StDev  SE Mean
1       6  0.773  0.162    0.066
2       6  0.630  0.172    0.070


Difference = μ (1) - μ (2)
Estimate for difference:  0.1427
95% CI for difference:  (-0.0718, 0.3572)
T-Test of difference = 0 (vs ≠): T-Value = 1.48  P-Value = 0.169  DF = 10
Both use Pooled StDev = 0.1668
```

**Appendix 2.6.1** – Regression analysis of Mannitol treated *Salmonella* growth vs time

## Regression Analysis: Mannitol versus Time

Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Regression | 1 | 0.088743 | 0.088743 | 2395.27 | 0.000 |
| Time | 1 | 0.088743 | 0.088743 | 2395.27 | 0.000 |
| Error | 12 | 0.000445 | 0.000037 | | |
| Total | 13 | 0.089187 | | | |

Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.0060868 | 99.50% | 99.46% | 99.27% |

Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | -0.22212 | 0.00922 | -24.08 | 0.000 | |
| Time | 0.039501 | 0.000807 | 48.94 | 0.000 | 1.00 |

Regression Equation

Mannitol = -0.22212 + 0.039501 Time

Fits and Diagnostics for Unusual Observations

| Obs | Mannitol | Fit | Resid | Std Resid | |
|---|---|---|---|---|---|
| 14 | 0.33767 | 0.35065 | -0.01298 | -2.47 | R |

R Large residual

**Appendix 2.6.2** – Regression analysis of sodium pyruvate treated *Salmonella* growth vs time

## Regression Analysis: SP versus Time

Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Regression | 1 | 0.070425 | 0.070425 | 2148.41 | 0.000 |
| Time | 1 | 0.070425 | 0.070425 | 2148.41 | 0.000 |
| Error | 12 | 0.000393 | 0.000033 | | |
| Total | 13 | 0.070818 | | | |

Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.0057254 | 99.44% | 99.40% | 99.17% |

Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | -0.20907 | 0.00868 | -24.10 | 0.000 | |
| Time | 0.035189 | 0.000759 | 46.35 | 0.000 | 1.00 |

Regression Equation

SP = -0.20907 + 0.035189 Time

Fits and Diagnostics for Unusual Observations

| Obs | SP | Fit | Resid | Std Resid | |
|---|---|---|---|---|---|
| 1 | 0.08300 | 0.07244 | 0.01056 | 2.14 | R |

R Large residual

**Appendix 2.6.3** – Regression analysis of sodium pyruvate treated *Salmonella* growth vs time

## Regression Analysis: PBS versus Time

Analysis of Variance

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Regression | 1 | 0.071141 | 0.071141 | 1439.84 | 0.000 |
| Time | 1 | 0.071141 | 0.071141 | 1439.84 | 0.000 |
| Error | 12 | 0.000593 | 0.000049 | | |
| Total | 13 | 0.071734 | | | |

Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|---|---|---|
| 0.0070291 | 99.17% | 99.10% | 98.81% |

Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|---|---|---|---|---|---|
| Constant | -0.1842 | 0.0107 | -17.29 | 0.000 | |
| Time | 0.035367 | 0.000932 | 37.95 | 0.000 | 1.00 |

Regression Equation

PBS = -0.1842 + 0.035367 Time

Fits and Diagnostics for Unusual Observations

| Obs | PBS | Fit | Resid | Std Resid | |
|---|---|---|---|---|---|
| 14 | 0.31492 | 0.32862 | -0.01370 | -2.26 | R |

R Large residual

**Appendix 2.6.4** – Summarised two sample t-test between *Salmonella* growth over time (mannitol treatment) vs. *Salmonella* growth over time (control)

## Two-Sample T-Test and CI

```
Sample   N     Mean    StDev  SE Mean
1       12  0.03950  0.00280  0.00081
2       12  0.03537  0.00323  0.00093


Difference = μ (1) - μ (2)
Estimate for difference:  0.00413
95% CI for difference:  (0.00158, 0.00669)
T-Test of difference = 0 (vs ≠): T-Value = 3.35  P-Value = 0.003  DF = 22
Both use Pooled StDev = 0.0030
```

**Appendix 2.6.4** – Summarised two sample t-test between *Salmonella* growth over time (sodium pyruvate treatment) vs. *Salmonella* growth over time (control)

## Two-Sample T-Test and CI

```
Sample   N     Mean    StDev  SE Mean
1       12  0.03519  0.00263  0.00076
2       12  0.03537  0.00323  0.00093


Difference = μ (1) - μ (2)
Estimate for difference:  -0.00018
95% CI for difference:  (-0.00267, 0.00231)
T-Test of difference = 0 (vs ≠): T-Value = -0.15  P-Value = 0.884  DF = 22
Both use Pooled StDev = 0.0029
```