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Antibacterial Activity of Commercially Available Plant Extracts on Selected Campylobacter jejuni Strains

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ABSTRACT

Campylobacter jejuni is recognized as a leading cause of foodborne illness worldwide with approximately 850,000 cases per year in the United Sates. A total of 18 species of *Campylobacter* have been identified worldwide to date. Foodborne transmission of *C. jejuni* is mainly through the consumption of unpasteurized milk, contaminated poultry meat and water. *C. jejuni* can survive in very diverse environments under stressful conditions in human and animals which is different from the specific conditions and media required in laboratory experiments. There has been increased research in recent years to identify naturally occurring antibacterial agents to control and eliminate *C. jejuni* on poultry. This study involves the evaluation of commercially available plant extracts of oregano, green tea and hawthorn to reduce /eliminate *C. jejuni* on poultry. Four isolates of *C. jejuni*, one human and three poultry isolates were used in this study. Research was done in broth culture to determine the effects of these extracts at different concentrations on the 4 isolates using cell counts at regular intervals of 0, 1, 2, 4 and 24 hours post-treatment. Results indicated that oregano and green tea were the most antibacterial and killed all the bacteria in broth culture in 24 hours at all concentrations tested. No significant differences were observed on the action of each of these plant extracts on the different isolates. Hawthorn was not successful in killing any of the strains of *C. jejuni* in 24 hours and no significant difference in cell counts post-treatment was observed compared to the control. The effects of these plant extracts then were tested as marinades on chicken breast fillets. The results showed that higher concentrations were required in the meat model study as compared to broth studies to completely kill all the bacteria. In the marinade studies, oregano was found to be the most effective, killing all 81176 and PRCC isolates in 2 hours and all the POCC and RECC *C. jejuni* isolates in 6 hours. The extracts of green tea and hawthorn tested were unsuccessful in

killing the *C. jejuni* isolates within 24 hours at all tested concentrations. The results of this study indicate the potential of using oregano extracts as antimicrobial against *C. jejuni* on poultry.

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DEDICATION

I would like to dedicate this work to my husband, Dr. Pradeep Nambiath for believing in me constantly motivating my journey towards the completion of this thesis.

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CHAPTER 1

INTRODUCTION

Campylobacter are gram-negative spiral shaped bacteria known for causing diseases in humans and animals (CDC 2014). These gram-negative bacteria are resistant to multiple drugs and are increasingly resistant to available antibiotics (CDC 2014). Campylobacteriosis is the most common disease caused by *Campylobacter*. Infection with *Campylobacter* produces antibodies that protect the individual against a second infection.

C. jejuni is one of the species of *Campylobacter* that is isolated from humans (Konkel *et* al, 2001) and is one of the most common causes of bacterial induced diarrheal disease worldwide (Zilbauer *et* al, 2008). The Center for Disease control and Prevention reports the occurrence of one case of *Campylobacter* in the United States for approximately 7000 persons in population (CDC 2014). There is active surveillance of such cases in the United Sates through Foodnet (CDC 2014). Symptoms of affected patients include diarrhea, fever and abdominal cramping with bacteremia and septic arthritis (Altekruse *et al*, 1999). *C. jejuni* infections are usually confined to the intestine, but a complication from this infection usually observed in a small percentage of cases is Guillain-Barré syndrome (GBS), a condition that can affect peripheral nerves (Zilbauer *et al*, 2008). Fluid and electrolyte replacement are regularly prescribed treatments for patients infected with campylobacteriosis (Altekruse *et al*, 1999). Treatment with antibiotics is recommended for patients with high fever and bloody diarrhea (Altekruse *et al*, 1999). A new trend being observed is the antibiotic resistant strains of *C. jejuni* that have been identified (Young *et al*, 2007). The United States Food and Drug Administration (FDA) has recently approved a conjugate vaccine for human trials that contain a polysaccharides from *C. jejuni* joined to a protein (Zuraw, 2014). Attempts similar to this underline the importance attached to the elimination of *C. jejuni* from food.

Human infection by *C. jejuni* can occur through a variety of routes with commercial food production being a primary source. Poultry is thought to account for 50-70% of *C. jejuni* infections (Epps *et al,* 2013) in humans also possibly through contaminated water and the formation of biofilms (Young *et al*, 2007). Poultry meat induced human infection can be controlled or prevented by a variety of methods which center around safe drinking water supply, biosecurity procedures and safe handling of poultry meat.

Campylobacter can potentially be controlled by the use of natural plant antimicrobials that are generally recognized as safe (Klančnik *et al*, 2012). Plant derived antimicrobials have also been investigated as components of hurdle technology as an alternative to synthetic antimicrobials. Compounds from plant sources such as essential oils, spices, plant extracts and their active components have shown their effectiveness against antibiotic resistant bacteria (Ravishankar *et al*, 2008). Plant based antimicrobials are effective because of certain key components such as phenolics like terpenes, aliphatic alcohols, aldehydes, ketones, acids and isoflavonoids (Tiwari *et al*, 2009; Hayek *et al*, 2013). Researchers have indicated that gram-negative bacteria are more resistant than gram-positive bacteria to natural antimicrobials (Davidson *et al*., 2013). Some non-phenolic constituents of essential oils are more effective against gram-negative bacteria (Hayek *et al*., 2013).

The main emphasis of this study is to determine the efficacy of commercially available oregano, green tea, hawthorn and curcumin plant extracts on reducing/eliminating 4 different strains of *Campylobacter jejuni* from poultry meat. Tea has been shown to inhibit the growth of *C. jejuni* strains within 4 hours (Diker *et al*, 1991) emphasizing their antimicrobial properties. Green tea composition is complex and consists of catechins, flavonoids, alkaloids, volatile oils, polysaccharides, amino acids, lipids, minerals and other compounds (Perumalla *et al*, 2011).

Comparing the antibacterial activities of different plant extracts on gram-positive and gram-negative bacteria, *Campylobacter* spp. was found to be more sensitive than other gram-negative bacteria to plant extracts (Klančnik *et al*, 2010).

Oregano essential oils have been shown to possess antimicrobial properties. However, this property of oregano differs with composition and is linked to geographical location, species and development stage of the plant (Falco *et al*, 2013). Oregano and some of its extracts have shown the ability to suppress the growth of gram-negative bacteria, gram-positive bacteria, yeast and fungi in *in vitro* models (Singletary, 2010). Oregano oil has been investigated in combination with vacuum packing and research has shown effectiveness against gram-negative bacteria and extended shelf life of chicken breast fillets by 8-9 days (Pavelkova *et al*, 2013).

Herbal medicine utilizes all components of the hawthorn plant (Chau, 2014). Hawthorn has shown strong inhibitory effect on *Staphylococcus aureus* and *Klebsiella pnuemoniae* with ethanol extract showing stronger antibacterial activity as compared to water extract (Niu *et al*, 2013). There is not a significant amount of literature on the effects of hawthorn extracts or its components on gram-negative and specifically *Campylobacter* spp. This study is aimed at learning more about the effect of hawthorn extract on *C. jejuni.*

JUSTIFICATION

In recent years there has been a strong consumer demand for quality meat products because of increased awareness and availability of information. *C. jejuni* is a gram-negative bacterium which is a leading cause of gastroenteritis from food. Poultry and other meat products are primary sources for this infection along with unpasteurized milk and contaminated water. According to official monitoring by the European Food Safety Authority in 2009, 90-100% of

raw chicken in retail market may be contaminated by *Campylobacter* (Piskernik *et al*, 2011). This study focuses on the use of commercially available plant antimicrobial extracts on the reduction/elimination of *C. jejuni*.

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Campylobacter jejuni is one of the most common bacterial causes of foodborne illnesses worldwide. Campylobacteriosis caused by *Campylobacter* is estimated to affect over 1.3 million people/ year with higher incidence in the summer months (CDC, 2014). In the United States over 99% of the infections associated with *Campylobacter* spp. are with *Campylobacter jejuni* (Friedman *et al.,* 2000). It is estimated that 76 people die from the disease every year (CDC, 2014). Severe diarrhea with stool specimens containing blood is associated with *Campylobacter* infections. These illnesses often are resolved without medication and the incubation period of *Campylobacter* in humans is usually between two to seven days. Deaths associated with the disease are rare and usually occur in very young or old people suffering from other diseases like AIDS (WHO, 2014). Treatment is not required except rehydration and electrolyte replacement (WHO, 2014; CDC, 2014). Guillain-Barré Syndrome (GBS), defined as an acute neuro-muscular paralysis, is a complication associated with *C. jejuni* infections (Altekruse *et al*., 1999). *C. jejuni* is microaerophilic (5% O_2, 10% CO_2 and 85% N_2), thermophilic, with an optimum growth temperature between 37^{o}C and 42^{o}C (Davis & DiRita, 2008).

2.2. History and Taxonomy of *Campylobacter*

The genus *Campylobacter* was suggested by Sebald and Veron for a “vibrio” which had been thought to be the cause for abortion in cattle by McFaydean and Stockman in 1913 (Veron and Chatelain, 1973). In 1886, Theodor Escherich recorded his observations of “spiral bacteria” diarrhea infected children (Altekruse *et al*., 1999) in “*Münchener Medizinische Wochenschrift”* (Butzler, 2004). The human strain *V. fetus* was isolated from patients as well as other “related vibrios” and their symptoms and infections were studied (King *et al.,* 1957). A common

symptom observed was the diarrheal symptoms in infected patients (Skirrow, *et al.,* 1977). The first major isolation techniques was reported in 1973 by Butzler which paved the way for successful characterization of the bacteria (Butzler, 2004). The "Vibrio like" bacteria were divided into four distinct species in the genus *Campylobacter* in work published by Veron and Chatelain in 1973. They were *C.fetus*, *Campylobacter coli*, *Campylobacter jejuni* and *Campylobacter sputorum* (Debruyne *et al., 2008*). There are 18 species of *Campylobacter* identified as - *C.fetus, C.hyointestinalis, C.lanienae, C.sputorum, C.mucosalis, C.concisus, C.curvus, C.rectus, C.gracilis, C.showae, C.hominis, C.jejuni, C.coli, C.lari, C.insulaenigrae, C. canadensis, C.upsaliensis* and *C.helveticus* (Debruyne *et al., 2008*). There are two subspecies within *C.jejuni,* C.*jejuni* subspecies *jejuni* and C.*jejuni* subspecies *doylei* (Epps *et al.,* 2013).

2.3. Morphology

Campylobacter are gram-negative, slender, spirally curved and non-spore forming rods with a single flagelllum at one or both ends of the cell responsible for its corkscrew like motion. The spiral form is usually observed in active cultures and indicates that the bacteria are actively growing (Svensson *et al.,* 2008). The coccoid form has been shown to be inactive and classified as old (NG *et al.,* 1985). The transition from spiral curved to the coccoid form is through the intermediate forms and can be identified via a double staining method (Alonso *et al*., 2002). Scanning electron microscope images have shown the existence of both types within a single colony with the active spiral form on the outside because of nutrient availability and coccoid in the middle (NG *et al.,* 1985). The transitional phase in between the two forms has been described as many researchers as being donut or spiral shaped possibly as a result of cell wall degeneration (Alonso *et al*., 2002; NG *et al.,* 1985; Svensson *et al*., 2008).

The transition to coccoid form is normally as a result of stress conditions, environmental conditions and lack of nutrients (Ikeda *et al.,* 2012). The coccoid form has characteristics similar to a viable but non-culturable (VBNC), state but researchers have accepted these to be separate phenomenon (Svensson *et al*., 2008). The success of *Campylobcater* existing in harsh environments can possibly be attributed to its dormant coccoid state although *C. jejuni* in VBNC state has been shown to be unable to return to a culturable state (Svensson *et al*., 2008).

2.4. Pathogenesis

2.4.1 Mechanism of pathogenesis

Diarrheal symptom is more prevalent in human infections as opposed to poultry infection (Young *et al.*, 2007). This behavior is very similar to that of *Escherichia coli* and control in the host is the most effective measure against the pathogen (Young *et al.,* 2007).The key to understanding the pathogenicity of *C. jejuni* is the understanding of the routes of entry into the host, the number of bacteria required to cause infection and the defense mechanism of the host once infection has been detected (Duan *et al.*, 2013). The pathogenesis factors can, therefore, be divided into host and pathogen dependent. Low doses of the organism have shown to cause illness (Black *et al.,* 1988) in experiments on humans. The symptoms of *Campylobacter* infection are inflammation, abdominal pain fever and diarrhea with an incubation period of 2-5 days (Blaser, et al., 1983). *C. jejuni* is able to achieve penetration of the mucus layer by virtue of its motility and unique morphology (Young *et al.*, 2007). This penetration was studied in humans (Van Spreeuwel *et al.*, 1985) and animals (Yao *et al.*, 1997) as well as *in vitro* experiments. The intestinal cells respond by initiating an inflammatory response (Epps *et al*., 2013) which is not common for all strains of *C. jejuni* (Young *et al.,* 2007). This can possibly be explained by how

different strains of *C. jejuni* access the epithelial cells differently (Nachamkin *et al.*, 2008). A model of pathogenesis proposed by Konkel *et al*., 2001, suggests protein synthesis through flagella as a means to interact with the host epithelial cells. It has been suggested that targeting adhesins specific to chicken epithelial cells may assist in the development of anti-*Campylobacter* drugs (Rubinchik *et al.*, 2012).

According to Epps *et* al., 2013, "The virulence of the pathogen is governed by motility, drug resistance, host cell adherence, host cell invasion, alteration of the host cell signaling pathways, induction of the host cell death, evasion of the host, immune system defenses and acquisition of iron , a micronutrient for growth". Additional determinant factors stated by Ketley *et al.,* 1997, are active secretion and epithelial disruption with leakage of serosal fluid and toxin production. Attachment and colonization requirements are chemotaxis, motility (Carrillo *et al.*,, 2004; Golden *et al.*, 2002; Grant *et al.*, 1993) and flagellar proteins secreted through flagella components (Konkel *et al.*, 2004; Song *et al.*, 2004). An important observation made by Ketley *et al.,* 1997 was that the different mechanisms are not necessarily operating in isolation but very dependent on the host and strain of infecting *C. jejuni*. After the onset of infection, severity, length and complications from the infection are a function of the age of the host and the immunity derived from previous *C. jejuni* infections (Altekruse *et al.*, 1999). The survival of *C. jejuni* in harsh environments points to the ability to adapt cell functions making it difficult to isolate its bacterial determinants (Nachamkin *et al.*, 2008).

2.4.2. Toxin production by *Campylobacter*

Cytolethal distending toxin (CDT) which causes "arrest at the G/S or G/M transition of the cell cycle" (Young *et al.*, 2007) is the only identified toxin produced by *Campylobcater* (Dasti *et al*.,

2010). This toxin is encoded by three genes *cdtA*, *cdtB* and *cdtC* (Miller, 2008) with the A and C responsible for the pathogen binding to the host cell (Dasti *et al*., 2010). Previously reported enterotoxin (Ketley, 1997; Ruiz-Palacios *et al*., 1983) has not been clearly identified from recent genome analysis (Hu & Kopecko, 2008). A summary is provided by Picket, 2000 describing CHO/HeLa cell cytotoxins, Vero-Active and Shiga-Like Toxins, hepatoxins, hemoslysins, CLRT, CLDT/CLRT and porin-lipopolysaccharide toxins.

2.4.3. Clinical manifestation

Campylobacter infections can occur without symptoms to severe and life threatening and are often self-limiting. *C. jejuni* and *C. coli* are the strains of *Campylobacter* spp. that cause infection in humans. In some cases complications can arise resulting in life threatening conditions (Zilbauer *et al.,* 2008). *C. jejuni* infections are exhibited in humans through severe gastroenteritis (Zilbauer *et al.,* 2008) with blood in the stool along with leukocytes (Epps *et al.,* 2013). In certain cases fever and abdominal cramping are also reported (Altekruse *et al.,* 1999). Genetic variations that exist between *C. jejuni* strains is an explanation offered for different symptoms seen in human infection (Dasti *et al.,* 2010). In severe case *C. jejuni* infections can cause reactive arthritis and an autoimmune disease Guillain-Barré syndrome (GBS) (Dasti *et al.,* 2010; Peterson *et al.,* 1994).

2.4.3.1. Gullian-Barré syndrome

Gullian-Barré syndrome (GBS) is a complication of a *C. jejuni* infection and presents as damage to the peripheral nervous system resulting in paralysis and in certain extreme cases death (Konkel *et al.,* 2001; Epps *et.al*., 2013) which can develop 1-3 weeks after *C. jejuni* infection. Gullian-Barré syndrome has been established as a mechanism where antibodies are the

pathogenic component that trigger Gullian-Barré syndrome (Shahrizaila *et al.*, 2010). Miller Fischer Syndrome is a subform of Gullian-Barré syndrome characterized by absent reflexes, inability to control muscle movements, and eye control problems (Epps *et al*., 2013). Two other forms are "acute inflammatory demyelinating neuropathy" and "acute motor axonal neuropathy" (Shahrizaila *et al.,* 2010). There had been concerns relating increased risk of GBS after vaccinations since 1976 when a 7-8 fold increased risk for GBS was observed in the 6 weeks after swine flu vaccination (Salmon *et al.,* 2013). This risk of GBS after administration of vaccines was not found to exist after an extensive study was conducted at the Northern California Kaiser Permanente site (Salmon *et al.,* 2013).

2.4.3.2. Reactive arthritis

Campylobacter induced intestinal infection can sometimes result in reactive arthritis (ReA) which can result in patients developing inflammation in the joints (Hannu *et al.,* 2002). This condition usually develops within four weeks of an infection with conditions lasting more than 6 months, a sign of severe infection (Pope *et al.,* 2007). Patients with HLA-B27 (human leukocyte antigen) positive condition is believed to interact with bacteria like *Salmonella* and *Campylobacter* causing this condition with reoccurrence reported as late as 7 years after the initial condition (Pope *et al.,* 2007). In a study in Finland, it was found that ReA had an annual incidence of around 4.3 per 100,000 with acute ReA at reduced levels with a higher percentage in adults (Hannu *et al.,* 2002). According to Townes, 2010; "Reactive arthritis is a concept, not a well-defined disease" with further study required to understand its prevalence in populations.

2.4.3.3. *Campylobacter* resistance mechanism

Campylobacter infections are usually self-limiting and rarely require treatment except in extreme cases and in patients whose immune systems are compromised. Typical antimicrobials like fluoroquinolones, macrolides, aminoglycosides and beta lactams have been noticed to be ineffective against many strains of *Campylobacter* (Iovine *et al.,* 2013). The similarity of *C. jejuni* infection symptoms with those caused by other bacterial infections results in treatments with fluoroquinolones (Iovine *et al.,* 2013). The resistance of *Campylobacter* can be related to the ability to mutate and acquire resistance by natural transformation, transduction or conjugation (Koluman *et al.,* 2012; Wieczorek *et al.,* 2013; Iovine *et al.,* 2013).

2.5. Epidemiology

In the early years of 1980's *Campylobacter* enteritis emerged as the most frequent form of bacterial diarrhea exceeding *Salmonella* infections (Skirrow, 1982). In studies in Norway published by Lassen & Kapperud in 1984; 3% of all severe intestinal illnesses were attributed to *Campylobacter* with infection isolated to travelers returning from abroad. A more recent study was carried out in England and Wales over 20 years showed a seasonal increase between May and June with increased incidence in areas with lower population density and lower percentage of native population (Nichols *et al.,* 2012). In the United States *Campylobacter* caused foodborne infection resulting in a total of 9135 illnesses from 262 outbreaks and three deaths in the period between 1997 to 2008 (Taylor *et al.,* 2013).

2.6. Reservoirs

There has been an increase in the number of outbreaks associated with *Campylobacter* but a decrease in *Campylobacter* infections was seen from 1997-1998 baseline (Taylor *et al.,* 2013). The transmission cycle of the bacteria from animals to humans is usually through the food chain.

Consumption, handling and cross-contamination of chicken meat are identified as the major contributors to *C. jejuni* infections along with drinking unpasteurized milk and contaminated water (Friedman *et al.,* 2000). *C. jejuni* mainly colonizes the cecum and colon of poultry with the colonization in broiler chicks causing transmission of the infection (Hermans *et al.,* 2011; Hermans *et al.,* 2012a; Epps *et al.,* 2013). Colonization of flock can take several weeks with cage free chickens taking 0-8 days (Horrocks *et al.,* 2009). Although poultry has been identified as the major animal reservoir for *C. jejuni*, cattle and other swine are known to carry the infection (Jensen *et al.,* 2006). Studies have suggested that *C. jejuni* and *C. coli* co-exist in pigs with *C.jejuni* always being in lower numbers (Jensen *et al.,* 2006; Madden *et al.,* 2000). Human infections can occur as a result of contaminated surface water with human to human transmission of infection not thought to be significant (Dasti *et al.,* 2010).

2.7. Growth conditions for *Campylobacter*

The inability of *C. jejuni* to survive and multiply outside of the host in normal atmospheric conditions was observed by to Park, 2002 (Murphy *et al.,* 2006). The bacterium grows best in low oxygen and is sensitive to harsh cold, dry and acidic environments (Altekruse *et al.,* 1999) and requires a microaerophilic growth environment (5% O2, 10% CO2, 85% N2) (Davis & DiRita, 2008). *C. jejuni* grows at a specific temperature range of 37ºC and 42ºC in select media (Davis & DiRita, 2008). The time required for growth of *C. jejuni* is also longer compared to other gram-negative bacteria like *E. coli* (Davis & DiRita, 2008). This growth outside of this temperature range can go down to zero within a few degrees (Hazeleger *et al.,* 1998) although survival at 7^{o}C has been recorded (Davis & DiRita, 2008). Another factor influencing its survival is the limited capability to utilize energy for survival and growth via carbohydrate fermentation, and adaptability is important for its survival in environment before host entry (Epps *et al.,* 2013).

A method of survival for *C. jejuni* is possibly with the formation of biofilms (Joshua *et al.,* 2006).

2.8. *Campylobacter* genome

The first *Campylobacter jejuni* strain (NCTC11168) was sequenced by Parkhill *et al.,* 2000 and since then 34 more have been completed (Zhou *et al.,* 2013). The genome of *Campylobacter jejuni* according to Gundogdu *et* al., 2007 is "1,641,481 bp long with 25 polymorphic regions" (Epps *et al.,* 2013). Strain variations and the work by Gundogdu *et* al., 2006 on NCTC11168 revealed "new information for 1450 of the original 1654 coding sequences" (Epps *et al.,* 2013). Stahl and Stintzi in 2011, identified one hundred ninety-five genes, essential to cell processes and core functions with genes grouped into categories based on function (Stahl *et al.,* 2011).

2.9. Prevention and control of *Campylobacter* in poultry

Prevention measures and reduction in colonization are different based on the stage of intervention in the colonization process. Prevention measures are responsible for reducing probability of poultry being colonized by *Campylobacter*, while the control of *C.jejuni* refers to the steps taken to reduce the presence of the pathogen prior to slaughter (Hermans *et al.,* 2011). Many studies to date suffer as a result of "poor design, sampling and statistical analysis" (Newell *et al.,* 2011). The control of *Campylobacter* in poultry can be divided into pre-harvest and post-harvest control strategies.

2.9.1. Pre-harvest control

Broiler chickens are the most common source for *Campylobacter* infection, so measures are needed to control pathogen colonization and spread of *Campylobacter* spp. in the flock. Studies

have shown that the spread of the pathogen can occur in the flock within three days of contact with the infected bird (Shanker *et al.,* 1990). Horizontal transmission of *Campylobacter* spp. can be controlled by "identifying the pathogen sources and farm management practices that result in exposure to the pathogen" (Doyle & Erickson, 2012). The important factors that influence the ability to control the colonization and spread of *Campylobacter* spp. in the flock are explained.

2.9.1.1. Biosecurity

The term "Biosecurity" can be broadly defined as steps or components of a program that prevent the entry of pathogens into the production site (Bagust *et al.,* 2013). Before explaining various steps required to achieve a biosecure environment, two terms require definition; vertical transmission and horizontal transmission. The term vertical transmission is used to define contamination of the egg within the hen prior to completion of the shell (Newell *et al.,* 2011). Horizontal transmission is the most widespread mode of spreading pathogen within the flock via chicks (Newell *et al.,* 2011). The most basic procedures include limited access to sites where poultry is stored, single point of entry and good hygiene (Bagust *et al.,* 2013). The presence of "hygiene barriers" has been shown to reduce risk of flock infection by 50% (Newell *et al.,* 2011). Important measures include washing hands, wearing protective clothing, disinfected boots and disinfection protocols (Ghareeb *et al.,* 2013). Implementation of biosecurity measures in Denmark decreased the *Campylobacter* recolonized flock, from 43% to 27% accompanied by a 12% reduction in human *Campylobacter* cases during the same period (Rosenquist *et al.,* 2009). Special attention has to be given to "environmental exposure" as increased levels can nullify biosecurity measures with delayed onset of flock colonization by *Campylobcater* (Ghareeb *et al.,* 2013).

2.9.1.2. Water treatment

Water being one of the reservoirs of *Campylobacter*, the proper treatment of drinking water can prevent the transmission of the pathogen to the flock. *Campylobacter* spp. can maintain long term contamination of water sources with survival time based on the state of the bacteria (Whiley *et al.,* 2013). There have been studies reporting efficiency of standard treatment processes successful in *Campylobcater* elimination although more resistant *C. jejuni* will require complicated treatment processes (Whiley *et al.,* 2013). Chlorination of water reduces the risk for *Campylobacter* colonization (Ellis-Iverson *et al.,* 2009). Ground water and drinking water are not considered to be major reservoirs for the pathogen although outbreaks in poultry and humans have been associated with these water supplies in the past (Whiley *et al.,* 2013).

2.9.1.3. Vaccination

The protective nature of "*Campylobacter*-specific antibodies" visible in young broilers during the initial weeks after hatch (Sahin *et al.,* 2003) suggests an effect of associated antibodies (Lin *et al.,* 2009). This supports the feasibility of vaccines for control of *Campylobacter* in poultry (Sahin *et al.,* 2003). This protection does not extend beyond the initial days and efforts to use purified and concentrated egg yolk antibodies from *C. jejuni* vaccinated hens has shown no significant reduction in *C. jejuni* colonies in poultry gut (Ghareeb *et al.,*2013). In spite of the improved protection offered by live vaccines, safety concerns have limited their use (Doyle & Erickson, 2012). The challenges faced by vaccine efficacy are strain, dose, age, breed, and administration (Barrow *et al*., 2007; Doyle & Erickson, 2012). *Campylobacter* colonization reduction in hosts is through "muscoal immunity" stimulation and to achieve this, "recombinant vaccines using attenuated live vaccines" can be administered (Kuttappan *et al.,* 2013). In

addition, subunit vaccines have shown promise whereas heterologous vaccines for *Campylobacter* have shown reduction in bacterial counts but not protection (Hermans *et al.,* 2012b). The diverse strains of *Campylobacter* limits the range of protection of standard vaccines (Kuttappan *et al.,* 2013) and, hence, no effective vaccine against *Campylobacter* is currently available (Hermans *et al.,* 2012b).

2.9.1.4. Prebiotics

According to Jordan *et al.,* 2014, "Prebiotics are defined as indigestible carbohydrates mostly with a relatively short chain length that can be fermented in the digestive tract". The principle involves using "live microbial feed" by which the harmless or good bacteria are encouraged to grow and control harmful pathogenic bacteria thereby improving intestinal health in poultry (Jordan *et al.,* 2014). In this method, individual strains are added or mixed with other strains which are cultivated separately (Ghareeb *et al.,* 2013; Doyle & Erickson, 2012). Currently, "mannanoligosaccharides" is only the organism based prebiotic in use (Ghareeb *et al.,* 2013; Jordan *et al.,* 2014).

2.9.1.5. Probiotics

Probiotic uses the technique where non-pathogenic pathogens are introduced into the animals and colonize the gastrointestinal tract of the animal and creating an inhospitable condition for foodborne pathogens (Doyle & Erickson, 2012; Ghareeb *et al.,* 2013; Jordan *et al.,* 2014). Bacterial strains of *Lactobaccilus, Enterococcus, Pediococcus* and *Bacillus* have shown promise in this application (Gaggia *et* al., 2010). The addition of probiotics and prebiotics, referred to as synbiotics, have shown to offer greater benefit together than when used individually (Awad *et al.,* 2009).

2.9.1.6. Bacteriocins

Bacteriocins are “ribosomal antimicrobial small peptides” generated by bacteria to inhibit other bacteria (Doyle & Erickson *et al.,* 2012; Lin *et al.,* 2009). They have been proposed as solutions to the antibiotic resistance of bacterial strains (Lin *et al.,* 2009). They have effects similar to antibiotics but differ in their synthesis, mode of action and resistance mechanism (Jordan *et al.,* 2014; Lin *et al.,* 2009; Doyle & Erickson *et al.,* 2012). These differences should make it safe to use in food applications (Cleveland *et* al., 2001). Lactic Acid Bacteria (LAB) and nisin are the two widely used bacteriocins in food applications (Cleveland *et al.,* 2001). BACTIBASE is an online database which is dedicated to bacteriocin research (Jordan *et al.,* 2014).

2.9.1.7. Bacteriophage

Bacteriophages are viral parasites that are function against pathogenic bacteria and can have a prolonged survival in host gut without negative effects (Jordan *et al.,* 2014). The bacteriophages function by reducing pathogen number and as a result multiply with researchers suggesting a minimum number of pathogens below which the bacteriophage will not function in this way (Doyle & Erickson, 2012; Jordan *et al.,* 2014). It is preferred to introduce bacteriophage into poultry before slaughter to prevent survival of bacteriophage resistant *Campylobacter* in flock (Ghareeb *et al.,* 2013). Research is required into the most effective ratio of bacteriophage to pathogen or the most effective mix of bacteriophage against the specific pathogen (Doyle & Erickson, 2012).

2.9.2. Post-harvest control

2.9.2.1. Preservation of fresh meat

The term fresh meat is used to describe freshly processed meat, vacuum sealed meat or atmosphere controlled meat. Meat is rich in nutrients that promote the growth of foodborne pathogens (Zhou *et al.,* 2010). Therefore, the primary purpose of preservation is to prevent the growth of pathogens and spoilage organisms that degrade the quality of meat (Zhou *et al.,* 2010). The most common preservation techniques are refrigeration, ionizing radiation, chemical preservation and preservation resulting from packaging (Zhou *et al.,* 2010). Moisture, another significant cause for pathogenic activity in meat needs to be controlled by drying or additive means to inhibit bacterial cell growth (Dave *et al.,* 2011).

2.9.2.2. Natural antimicrobials for control of *Campylobacter* in poultry

The health impacts of using synthetic food additives that have long term side effects to reduce the activity of pathogens has been under significant review in the last decade (Witkowska *et al.,* 2013). The importance of natural and organic ingredients that do not trigger food allergies and the information on negative side effects of increased sodium intake has also increased interest (Davidson *et al.,* 2013 ; Tajkarimi *et al.,* 2010 ; Burt *et al.,* 2004 ; Jayasena *et al.,* 2013; Witkowska *et al.,* 2013). Consumers across the world have indicated a preference for “natural food additives” with the ability to act against a wider range of pathogens (Jayasena *et al.,* 2013). Natural antimicrobials from plant or animal sources have to meet the conditions of non-toxicity, affordability, low concentration inhibition and neutral to taste and smell (Davidson *et al.,* 2013). Naturally occurring additives that have been investigated for antimicrobial properties include essential oils, nisin, chitosan and lysozyme (Zhou *et al.,* 2010). The preservation ability of chitosan through penetration of the outer cell membrane of microbes has been investigated (Siripatrawan *et al.,* 2012).

2.9.2.3. Plant sources

Phenolic compounds, known for their antimicrobial effects are the main antimicrobial agents in plants (Hayek *et al.,* 2013). Steam distillation, hydro distillation and supercritical fluid extraction are the most common extraction methods for large scale plant antimicrobial production (Tajkarimi *et al.,* 2010). Commercial products have also been produced using bioengineering (Burt *et al.,* 2004). According to Tiwari *et* al., 2009, "the antimicrobial compounds in plant materials are usually found in essential oil fraction of the leaves, flowers, bulbs, seeds, fruits and other parts of the plant". The constituents of these plant components that are used to fight microbes are polyphenols, quinones and alkaloids (Lorenzo *et al.,* 2014).

Phenols are classified into three groups: simple phenols, phenolic acids, hydroxycinnamic acid derivatives with the latter group consisting of catechins, proanthocyanins, anthocyanidins, flavons, flavonols and their glycosides (Smid *et al.,* 1999). The role of phenols in plant defense is still unclear with the phenolic compounds thought to affect the cytoplasmic membrane of pathogens changing its structure and function (Witkowska *et al.,* 2013; Holley *et al.,* 2005). The presence of multiple phenolic compounds in extracts would involve multiple modes of action against the bacteria (Witkowska *et al.,* 2013; Holley *et al.,* 2005). The effect of phenols vary with concentration; lower concentrations affecting the activity of enzymes and higher concentration causing protein denaturation (Tiwari *et al.,* 2009).

2.9.2.4. Spices and essential oils (EO)

Research has identified plants with different antimicrobial action depending on the type and strain of bacteria but the mechanism of action of only a small fraction of the essential oils (EO) is well documented (Burt, 2004). Gram-positive bacteria are generally more sensitive to essential

oils than gram-negative bacteria because of the outer membrane present in gram-negative bacteria though *Campylobacter* spp. is a more sensitive gram-negative bacterium (Burt *et al.,* 2004; Holley *et al.,* 2005; Klančnik *et al.,* 2010). The possibility of this defense mechanism in gram-negative bacteria just delaying the action of EO's has been studied by researchers (Fischer *et al.,* 2008). Essential oil compounds are classified into a group containing terpenes and trepenoids and a group containing aromatic compounds (Jayasena *et al.,* 2013). The EO's from clove, thyme, rosemary, ginger and oregano on meat and meat products have been summarized by Jayasena *et al.*, 2013 showing significant inhibition of bacterial pathogens. The antibacterial activity of essential oils is attributed to the presence of phenols like carvacrol, eugenol and thymol (Akthar *et al.,* 2014; Oussalah *et al.,* 2006).

Mechanism of antimicrobial action

The major antimicrobial action of EO is attributed to its "hydrophobic" nature of certain compounds which penetrate through the outer membrane (Krisch *et al.,* 2008). The research into EO components has identified the action of the components on the cytoplasmic membrane of the pathogen (Burt *et al.,* 2004; Davidson *et al.,* 2013; Lv *et al.,* 2011). According to Davidson *et al.,* 2013, "increased membrane permeability, dissipation of proton motive force, inhibition of ATP synthesis and enzyme inhibition" are factors in the inhibitory mechanism of EO (Tajkarimi *et al.,* 2010; Akthar *et al.,* 2014). Terpenoids which are modifications of terpenes have shown antibacterial activity over a wide range of pathogens (Hyldgaard *et al.,* 2012).

However, the effectiveness of antimicrobials has been shown to decrease in the food model as compared to *in vitro* (Lv *et al.,* 2011). Research has revealed that a combination of different natural antimicrobials against pathogenic bacteria has an additive and combined effect

preventing the pathogens from building resistance (Škrinjar *et al.,* 2009; Bassole *et al.,* 2012; Techathuvanan *et al.,* 2014). This combination effect can be between molecules, sections of plant or different plant varieties (Vuuren *et al.,* 2011).

2.9.2.5. Essential oils in hurdle technology

According to Leistner *et al.,* 2000, "hurdle technology is used in industrialized as well as developing countries for the gentle, but effective preservation of food" ,the most important of which are temperature, water activity, acidity, redox potential, preservatives and competitive microorganism (Leistner *et al.,* 2000). Essential oil added to edible films and coating is an example of the role plant based antimicrobials could play in hurdle technology (Jayasena *et al.,* 2013).

2.9.2.6. Plant extracts

Oregano

Oregano is a common name for a plant genera usually used to reference *Origanum* and there are at least "61 species of 17 genera that belong to six families" under the name oregano (Kintzios S, 2003). The volatile oils are the most important group of chemicals isolated form oregano with composition of oils varying significantly among different types of oregano with phenolic monoterpenoids such as carvacol and thymol being significant components of oregano (Kintzios S, 2003). The composition of carvacol and thymol, two major antibacterial components of oregano can vary depending on the region where it is grown as well as the time the crop is picked (Burt, 2004). There have been differences observed in the antibacterial activities of EO's between studies on the same bacteria. Studies on the effect of oregano oil against *S.Typhimurium* in tests by Mith *et al.,* 2014 was found to be approx. 2-10 times that observed by Hammer *et* al.,

1999. The inhibitory effect under “modified atmospheric packaging” containing oregano on the same bacteria was observed by Sakandamis *et al.,* (2002) in “sterile and naturally contaminated beef fillets” (Akthar *et al.,* 2014). During an analysis of the different tests using essential oils, Hood *et* al., (2003) observed the uneven distribution of essential oils in the medium could possibly lead to higher concentration zones and possibly inaccurate results. Antimicrobial activity of oregano is primarily against bacteria and fungus (Kintzios S, 2003). *O. vulgare* was shown to yield one of the most effective oils among the 52 investigated EO’s according to Hammer *et al.*, 1999 with Biondi *et al.,* 1993 and Izzo *et al.,* 1995 reporting strong inhibitory action gram-positive and gram-negative bacteria. This action has been attributed to the effect of thymol and carvacol (Kintzios S, 2003). In context of determining antibacterial activity, the definition of the term minimum inhibitory concentration (MIC) is very important. MIC according to Cosentino *et al.,* 1999 can be defined as the lowest concentration resulting in a significant decrease in inoculum viability (Burt, 2004). The MIC values of thymol and carvacol has been reported by researchers against a variety of gram-positive and gram-negative bacteria (Cosentino *et al.,* 1999, Kim *et al.,* 1995; Lambert *et al.,* 2001; Pol *et al.,* 1999). Thymol and carvacrol, constituents of oregano are believed to show antibacterial effects because of the ability to penetrate the cell and interfere with cellular functions according to Lambert *et al.,* (2001), Marino *et al.,* (2001) and Karami-Osboo *et al.,* (2010) and “disturb the active site of enzymes” (Kotan *et al.,* 2013).

Oregano has shown to delay the start of spoilage by Chouliara *et al.,* 2007 used with “modified atmospheric packaging” (Tajkarimi *et al.,* 2010). Oregano essential oil enriched chotisan films have shown strong inhibitory effect against gram-positive *Listeria monocytogenes* and gram-negative *Escherichia coli* (Zivanovic *et al.,* 2005). The difference in effectiveness against gram-

positive and negative bacteria is due to their “hydrophobic nature” of the essential oil (Dussault *et al.,* 2014) with the lipopolysaccharide component of the outer membrane of gram-negative bacteria (Dussault *et al.,* 2014; Du *et al.,* 2009). The formation of biofilms in bacteria has been shown to be influenced by carvacrol interfering with bacterial quorum sensing ability (Burt *et al.,* 2014).

Green tea

Tea can be classified into three types on the basis of their fermentation process; green tea, oolong tea and black tea (Wang *et al.,* 2000). Green tea is made from the leaves of *Camellia sinensis* and is prepared by preventing the “enzymatic oxidation of catechins” by firing or by steaming (Wang *et al.,* 2000). The major constituent of green tea is polyphenols of which flavonoids is believed to have health benefits (Taylor *et al.,* 2005). Polyphenols like flavan-3-ols, flavonols and tannins have shown higher antimicrobial activity as compared to others and also work well with antibiotics, Figure 1 (Daglia, 2012).

Phenolic acids, flavonoids and lignans are the classification of polyphenols occurring in plants with structural binding and phenol rings being the major difference between them (Gharras *et al.,* 2009). The major benefit of polyphenols is their antioxidant activity with genetic and environmental factors affecting the amount of polyphenols present in fruits and vegetables (Gharras *et al.,* 2009). Polyphenols account for a third of composition of green tea (An et al., 2004). The major polyphenols found in green tea are epicatechin (EC), gallocatechin (GC), gallocatechin gallate (GCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCg) (Cooper *et al*., 2005).

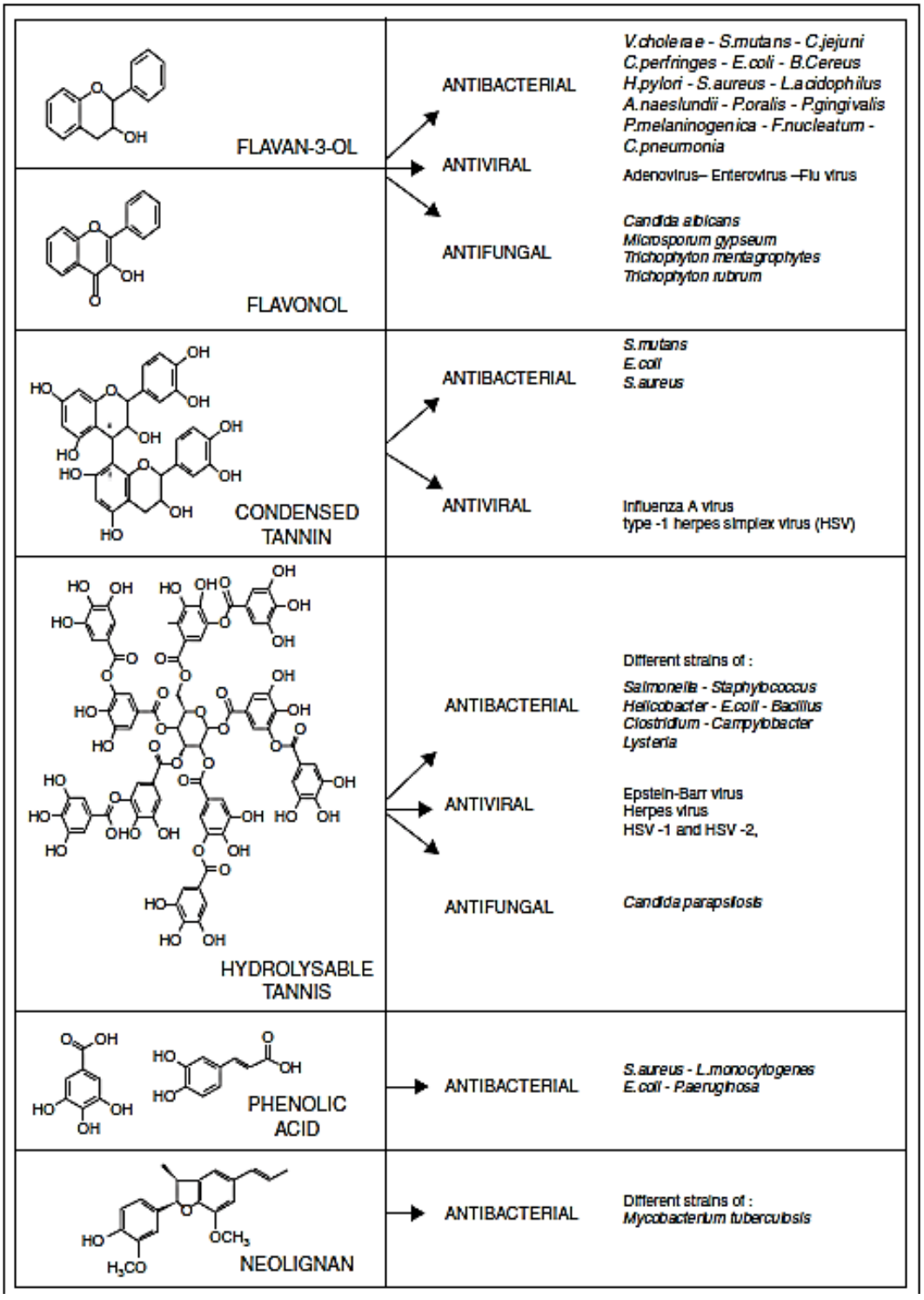

Figure 2.1: Chemical structure of polyphenol classes and the microorganisms sensitive to them (Source: Daglia / Current opinion in biotechnology, 23(2), 174-181).

In studies by Yam *et al*., 1997, showed that “gallocatechins and their gallates” are the main components that are responsible for its antibacterial activity. The plant kingdom has flavonoids that help in fighting fungal microbes and protect against radiation (Harborne *et al.,* 2000; Middleton *et al.,* 1994; Cushnie & Lamb, 2005). The major functions of this class of compounds are related to energy acquisition from the environment, basic growth and energy transfer (Harborne *et al.,* 2000; Middleton *et al.,* 1994; Cushnie & Lamb, 2005). Flavonoids can be divided into flavones, flavanones, catechins and anthocyanins on the basis of molecular structure (Nijveldt *et al.,* 2001). The flavonoids in their common forms are “water-soluble” and “less reactive towards free radicals” (Rice-Evans *et al.,* 1997). In a study of antibacterial activity of flavonoids against methicillin-resistant *S. aureus* strains, it was found that an increase in flavonoid concentration resulted in decreased bacterial count and the structure of flavonoids affected the activity against *S. aureus* (Alcaraz *et al.,* 2000).

Flavan-3-ols are shown to be the most significant phenolics in green tea accounting for over two thirds of its antioxidant activity (Stewart *et al.,* 2005). Ethanol has shown to be a more effective extraction medium as compared to water for antioxidative research in studies related to green tea with higher polyphenol content (Gramza *et al.,* 2006). This effect was also seen in antibacterial studies of green tea extract against *Listeria monocytogenes* in methanol and aqueous extract where methanol showed increased antibacterial activity (Mbata *et al.,* 2008).

According to Shimamura *et al.,* 2007, “The antibacterial activity of catechins is considered non-specific with limited species selectivity”. Research by Hammilton-Miller (1997) and Yoda (2004) have revealed mode of interaction to EGCg by gram-negative bacteria like *E.coli* and gram-positive bacteria like *S.aureus* (Shimamura *et al.,* 2007) and also observed antibacterial activity of ECG and EGCg (Miller, 1995). The antimicrobial action of tea extracts were shown to

be different between gram-negative and gram-positive bacteria in a study against different pathogenic bacteria where *Staphylococci* and *Y. enterocolitica* were the most sensitive gram-positive and gram-negative bacteria respectively (Yam *et al.,* 1997). Tests that were conducted to compare the antibacterial activity of Chinese green tea crude extracts against different bacteria showed more potency against *S.aureus* and *L.monocytogenes* than *Salmonella* and *E.coli* (Si *et al.,* 2006). In studies conducted on Mongolian gerbils, the catechin ECG was shown to eradicate 10-36% (depending on the diet combination fed to the animals) of the treated gerbils (Mabe *et al.,* 1999). According to Mabe *et al.,* 1999, "EGC has demonstrated ability to inhibit the urease activity and motility of *H.pylori*". Research conducted by Friedman *et al.,* 2006 involving evaluation of antimicrobial activity of green tea catechins against *Bacillus cereus* revealed that "most compounds were more active than were medicinal antibiotics such as tetracycline and vancomycin at comparable concentrations". Other applications of green tea extracts include antiseptic creams, mouthwashes (Taylor *et al.,* 2005) and introduction to vacuum cleaner filters to reduce airborne contaminations (Hara, 2001).

Hawthorn

Hawthorn has been extensively used as medicinal material for their health effects and antioxidant activity in parts of Europe and China for hundreds of years (Liu, 2012). The *Crataegus* species commonly referred to as hawthorn has over 1000 species native to Asia, Europe and North America (Zhao and Tian, 1996). Hawthorn plants have been shown to contain procyanidins, flavanols, flavonols. *C*-glycosyl flavones, phenolic acids, anthocyanins and lignans (Liu, 2012) having astringent, antispasmodic, cardiotonic, diuretic, hypotensive and antiatherosclerotic properties (Tadić *et al.,* 2008). Hawthorn extracts are consumed as tea, tinctures or fruits in Europe at upto 720 mg/day (Benli *et al.,* 2008).

In a study conducted on rats using hawthorn extract against *Micrococcus flavus*, *Bacillus subtilis* and *Lysteria monocytogenes* showed "moderate bacterial acivity" and no effect against *Candida albicans* (Tadić *et al.,* 2008). Antibacterial activity of ethanol extracts of *Crataegus monogyna* (Hawthorn) against *S.aureus* and *E.coli* and *P.aeruginosa* was observed using disk agar diffusion method (Ignat *et al.,* 2013). A similar difference in antibacterial activity between ethanolic and water extracts of hawthorn on *S.aureus* and *Klebsiella pneumonia* was also observed (Niu *et al.,* 2013). An observation by Niu *et al.,* 2013, was that the higher percentage of gallic acid content present in the ethanol extract over water extract might explain the difference in antibacterial activity.

In studies using *C. tanacetifolia*, a *Carataegusi* species from Turkey, antibacterial effects were observed against *Bacillus subtilis*, *Shigella*, *S.aureus* and *L.monocytogenes* (Benli *et al.,* 2008). Ethanol extracts of *Crataegus oxyacantha,* another variant of hawthorn, was investigated and shown ineffective against *B.subtilis* and *S.aureus* but effective against *E.coli*, *P.aeruginosa* and *S.albony* (Kostić *et al.,* 2012). The "flavonoid structure" was found to influence effectivity against different bacteria (Kostic *et al.,* 2012). The antibacterial activity of *C.azarolus* variety against *S.aureus*, *E.faecalis*, *E.coli, S.enteritidis and S.typhimurium* bacteria showed varying degrees of inhibition (Nadia *et al.,* 2014). However, it was reported that *C.monogyna* and *C.oxycantha* berries in equal ratio in ethanol, similar to the mix found in commercially available extracts, showed moderate antibacterial activity against *M.flavis*, *B.subtilis* and *L.monocytogenes* and no effect against *C.albicans* (Tadic *et al.,* 2008). In studies on the effect of *Crataegus monogyna* on *C.jejuni* (clinical isolate), the extract did not totally inhibit the growth of the bacterium but less than 25% growth was observed and the results were consistent for both water

and methanol extracts (Galgóczy *et al.,* 2009). *Cartaegus pinnatifada,* a species found in China has been used in native medicine and also combined with other herbs (Wu *et al.,* 2014).

Curcumin

Curcumin is responsible for the yellow color of turmeric (Chattopadhyay *et al.,* 2004). In 1815, Vogel and Pelletier isolated curcumin (Roughley *et al*., 1973). Its chemical structure was explained in 1973 (Roughley *et al*., 1973). In 1995, Ruby *et* al., stated curcumin to consist of 94% curcumin I and 6% of curcumon II and 0.3% of curcumin III (Chattopadhyay *et al.,* 2004). Human clinical trials have shown that curcumin has no toxicity at doses of 1-10 grams/day (Wu, 2003). The geographical source of curcumin has shown to play an important role in resistance to growth of bacteria (Naz *et al.,* 2010). In studies involving comparison of antibacterial effects of microcapsule curcumin against *S.aureus, B.subtilis, B.cereus, E.coli* and *Y.enterocolitica*, results were comparable to free curcumin (Wang *et al.,* 2009).

Curcumin has been found to help in control of *E.coli*, *P.aeruginosa*, *Proteus mirabilis* and *Serratia marcescens* by limiting the ability of these pathogens to form biofilms via quorum sensing (Packiavathy *et al.,* 2014). The negative influence of curcumin on *H.pylori* biofilm formation was verified in studies by Pattiyathanee *et al.,* 2009. This antimicrobial ability of curcumin against *H.pylori* was seen in infected mice and not dependent on the strain that caused the infection (De *et al.,* 2009). Curcumin was found to control cell division in *Bacillus subtilis*, via filamentation (Rai et al., 2008).

Aqueous extracts of *Curcuma longa* from the *Zingineraceae* family had shown activity against *E. coli, S. aureus, K. pneumoniae* and *S. epidermidis* at low concentrations (Niamsa and Sittiwet, 2009). In a study of the effect of *C.longa* extracts against methicillin-resistant *S. aureus*, ethyl

acetate extract of *C.longa* showed greater antibacterial action as compared to methanol or aqueous extracts (Kim *et al.,* 2005). This is in agreement with other studies that showed the increased antibacterial activity of ethanolic extracts of *Curcuma longa* over aqueous extracts (Harit *et al.,* 2013).

Synthetic modifications of curcumin like indium curcumin have shown antibacterial effects superior to curcumin in *in vitro* studies (Tajbakhsh *et al.,* 2008). Bioconjugate and metal-conjugate analogues have also been synthesized to improve the antibacterial activity of curcumin (Marathe *et al.,* 2011).

2.9.2.7. Effect of plant antimicrobial marinade on survival of *Campylobacter* in meat

There is a significant increase in requirement of marinated broiler meat in some countries with taste and ease of cooking driving this need (Birk *et al.,* 2010). The antibacterial effect of such marinades is an added benefit (Birk *et al.,* 2010). Recent studies on the effect of thyme orange essential oil combination showed approx. 3 log cfu/ml reduction in *C. coli* on the surface of breast fillets as compared to controls (Thanissery *et al.,* 2014). A reduction of *C. jejuni* was observed over 35 days when using 3% cultured sugar/vinegar blend on precooked chicken (Park *et al.,* 2014).

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CHAPTER 3

ANTIBACTERIAL ACTIVITY OF COMMERCIALLY AVAILABLE PLANT EXTRACTS ON SELECTED *CAMPYLOBACTER JEJUNI* STRAINS

ANTIBACTERIAL ACTIVITY OF COMMERCIALLY AVAILABLE PLANT EXTRACTS ON SELECTED *CAMPYLOBACTER JEJUNI* STRAINS

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ABSTRACT

Campylobacter jejuni causes over 99% of the foodborne infections associated with *Campylobacter* in the United States. This study involves evaluation of commercially available plant extracts of oregano, green tea, hawthorn and curcumin against four isolates of *C. jejuni*. Initial studies were carried out in broth cultures to determine the effectiveness of the extracts. Then, the study was carried out on chicken breast meat. Cell counts were determined at intervals of 0, 2, 6 and 24 hours and bacterial viability was determined using different concentrations of the above extracts. Both oregano and green tea extracts were found to be antibacterial in broth cultures that killed all bacteria at 24 hours. As marinades, the extracts were found to be effective only at high concentrations. No significant differences were found in the antibacterial effects of the extracts on different *C. jejuni* strains. These results demonstrate that commercially available plant extracts such as oregano and green tea have potential to reduce and/or eliminate *C. jejuni* in chicken meat.

KEYWORDS: *C. jejuni*, Green tea, Oregano, Hawthorn, Curcumin, Plant Extracts

INTRODUCTION

Foodborne illnesses are a cause of concern for both developed and developing countries requiring food security to be carefully monitored by governments as populations grow and average life spans increase. In such an environment antimicrobials added to foods and used in food marinades have become important in reducing bacterial activity (Davidson *et al.*, 2013). To reduce bacterial loads on meat is especially challenging because of the rich nutritional availability, pH and water activity level (Jayasena & Jo, 2013).

Campylobacter spp. is one of the major causes of foodborne illnesses in the world. Although symptoms of this condition are usually not severe, the total annual cost as a result of these infections is millions of dollars in the United States. Investigations have revealed significant links between human *Campylobacter jejuni* infections and the consumption of undercooked poultry (Altekruse *et al.*, 1999). Poultry products are an important component of the human diet and the safety of poultry products is very important (Venkitanarayanan *et al.*, 2013). In an opinion expressed by the European Food Safety Authority (EFSA), the European Center for Disease Control and Prevention (ECDC), the European Medicines Agency (EMEA) and European Commissions Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), the importance of *Campylobacter* and chicken meat production was highlighted (Možina *et al.*, 2011).

Natural antimicrobials can be found in plant or animal sources with plant sources the more significant which can be used in the form of pure compounds or extracts. Phenolics, phenolic acids, quinones, flavonoids, thiosulfinates. glucosinolates and tannins that exist in these plant products are responsible for antibacterial properties of the plants (Witkowska *et* al., 2013; Hayek

et al., 2013). These compounds are found in different parts of the plant from the leaves and fruit to the root, i.e., all parts of the plant and are responsible for their natural ability to protect against insects and harmful pathogens (Davidson *et al.,* 2013). The major mechanism of action of essential oils has been explained by Burt, (2004) by its ability to significantly weaken the cytoplasmic membrane resulting in leakage of cell contents and bacterial cell destruction.

Oregano (*Origanum vulgare* L.) has components that have shown varying degrees of antimicrobial activity which is dependent on the species, geographical location, age and component of the plant from which the extract is separated (Burt, 2004). The major components along with their identified range of percentage were compiled by Jayasena & Jo, (2013). Thymol and eugenol are the major components with inhibitory action against a wide range of bacteria with *p*-cymene being a minor component of oregano. *In vitro* model study using cecal contents, carvacol at 0.75% and thymol at 1% resulted in significant reduction of *C. jejuni* populations after 24 hours (Venkitnarayanan *et al.,* 2013).

The antibacterial activity of tea against *C. jejuni* and *C. coli* has been reported with black and green tea extracts achieving total inhibition at 4 hours (Diker *et al.,* 1991). There have been conflicting reports detailing the effectiveness of tea which could be because the researchers used different types of tea for experiments along with different sources and strengths of tea in the study (Miller 1995). Flavonoids are important secondary organic compounds (Rice-Evans *et al.,* 1997) of which flavanols and flavonols are important subgroups found in tea (Wang *et al.,* 2000). Important constituents of green tea are catechins (Wang *et al.,* 2000) which have shown significant antibacterial activity and constitutes 15% of the dry weight of green tea (Mabe *et al.,* 1999) with epigallocatechin gallate (EGCg) being the major antimicrobial agent (Miller, 1995). Theaflavins and thearubigenes are other groups present in tea leaves (Gramza *et al.,* 2006). An

important study by Friedman *et al*., 2006, evaluated the antimicrobial activities of tea catechins against *B.cereus* and documented the ratio in antibacterial activity between the most active catechin and the least active catechin.

Hawthorn has been used for centuries as part of medicines across Europe and Asia as is referred to as the *Crataegus* species (Zhao and Tian, 1996). Hawthorn extracts are normally consumed as tea or tinctures (Benli *et al.,* 2008). Hawthorn extract has shown antibacterial activity against *Micrococcus flavus, Bacillus subtilis* and *Lysteria monocytogenes* with no effect against *Candida albicans* (Tadić *et al.,* 2008). In studies on the effect of *Crataegus monogyna* on *C. jejuni*, the extract did not totally inhibit the growth of the bacterium but less than 25% growth was observed and the results were consistent for both water and methanol extracts (Galgóczy *et al.,* 2009).

Curcumin is responsible for the yellow color of turmeric and comprised of 94% curcumin I, 6% of curcumin II and 0.3% of curcumin III (Chattopadhyay *et al.,* 2004). *Curcuma longa* has shown antibacterial activity against *E. coli, S. aureus, K. pneumoniae* and *S. epidermidis* at low concentrations (Niamsa and Sittiwet, 2009). In studies, microcapsule curcumin had antibacterial effects comparable to free curcumin against *S. aureus, B. subtilis*, *B. cereus*, *E. coli* and *Y. enterocolitica* with *S. aureus* inhibition the highest and *E. coli* the lowest (Wang *et al.,* 2009).

MATERIALS AND METHODS

Bacterial strains and growth media:

In this study, four different *C. jejuni* strains were used: one human strain (81176) and three poultry strains isolated from different stages of poultry processing such as a pre-chilled chicken carcass (PRCC), a post-chilled chicken carcass (POCC) and a retail chicken carcass (RECC). Media used for initial culturing of *C. jejuni* strains was *Campylobacter* Enrichment (CE) broth

(Acumedia®). Frozen stock cultures of 81176, PRCC, POCC and RECC were passed twice onto *Campylobacter* blood agar plates and were then inoculated into CE broth which was incubated at 42ºC under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) for 24 hours. *Campylobacter* Enrichment Agar (CEA) supplemented with 5% horse blood was used for plating the cultures after serial dilution.

Plant extracts:

The four plant extracts used in this study were green tea, oregano, hawthorn and curcumin. All were commercially purchased from gaia® herbs except for curcumin. Curcumin in powder form was purchased from Alfa Aesar®. The constituents of the plants were extracted using either water or a combination of water and alcohol in a ratio to maximize constituent content ("Right+Right," 2014). The next step in the extraction process used 24 millibar pressure at 60°C to concentrate the extract via a closed loop system maintaining integrity of all components ("Low+Low," 2014).

Green tea leaf extract:

The organic green tea liquid herb extract was in water with 500 mg/ml herb equivalency.

Oregano leaf extract:

The oregano leaf extract was from the herb *Origanum vulgare* with a 333mg/ml herb equivalency.

Hawthorn extract:

The extract purchased was of the hawthorn berry, flower and leaf of *Crataegus* spp with 667mg/ml herb equivalency.

Curcumin extract:

The extract has a total of 95% curcuminoid content and was derived from turmeric rhizome.

These extracts were diluted in 1X phosphate buffered saline (PBS) to obtain concentrations of 0.25%, 0.5% and 1% of the purchased extracts for the broth culture studies as well as 1%, 5%, 10%, 20%, 30%, 50% and 100% of the purchased extracts for chicken meat model studies.

Preparation of plant extracts:

***Broth culture study*:**

A 0.25% of the purchased extract concentration was obtained by diluting 0.025 mL of the extract in 9.975 mL PBS while a 0.5% of the purchased extract concentration was obtained by diluting 0.05 mL of the extract in 9.95 mL PBS; a 1% of the purchased extract concentration was obtained by diluting 0.1 mL of the extract in 9.9 mL PBS.

In chicken meat model study, a total volume of 30 mL extract was prepared as follows:

- A 1% of the purchased extract concentration was obtained by diluting 0.3 mL of the extract in 29.7 mL of PBS.
- A 5% of the purchased extract concentration was obtained by diluting 1.5 mL of the extract in 28.5 mL of PBS.
- A 10% of the purchased extract concentration was obtained by diluting 3 mL of the extract in 27 mL of PBS.
- A 20% of the purchased extract concentration was obtained by diluting 6 mL of the extract in 24 mL of PBS.

- A 30% of the purchased extract concentration was obtained by diluting 9 ml of the extract in 21 mL of PBS.
- A 50% of the purchased extract concentration was obtained by diluting 15 mL of the extract in 15 mL of PBS.
- A 100% of the purchased extract concentration was obtained by using 30 mL of the extract undiluted.

***Effect of plant extracts on C. jejuni strains in broth cultures*:**

All the plant extracts green tea, oregano and hawthorn were tested at 3 different concentrations of 0.25%, 0.5% and 1% of the purchased extract concentration. The extracts were prepared in 1X phosphate buffered saline (PBS) solution. To each 1 mL of the extracts, 100 µL of each of the *C. jejuni* strains was added.

A volume of 1.1 mL of each of the cultures was used as positive control. The treatment culture tubes along with the controls were incubated at 42ºC under microaerophilic conditions. Samples were taken at time intervals of 0h, 1 h, 2h, 4h and 24h and subsequent serial dilutions were performed in 1X PBS from 1 to 7. 100 µL of each of the treated cultures were plated onto *Campylobacter* Enrichment Agar plates supplemented with 5% horse blood. All these plates were then incubated at 42ºC under microaerophilic conditions for 48 hours and viable *C. jejuni* colonies were counted using a colony counter. The results were recorded and expressed in terms of log CFU/mL vs time. The experiments were performed in triplicates.

Meat model studies:

Preparation of marinade:

Marinades used were prepared using one of the three plant extracts of oregano, green tea and hawthorn. Marinades of green tea and hawthorn extracts were used at concentrations of 50% and 100% of the purchased extract concentration whereas marinade with oregano was prepared at 30% and 50% of the purchased extract concentration. All the marinades were prepared on the same day of the experiment by diluting the extracts with 1X phosphate buffered saline solution (PBS).

Preparation of inoculum

Frozen stock cultures of 81176, PRCC, POCC and RECC were passed twice onto *Campylobacter* blood agar plates and were then inoculated into *Campylobacter* Enrichment (CE) broth which was incubated at 42ºC under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) for 24 hours.

A chicken meat model was used in this study using uncooked boneless chicken breasts fillets purchased from a local supermarket in Fayetteville, Arkansas. The breast fillets were cut into approximately 1 inch square pieces using a sterile knife under a laminar flow hood to prevent external contamination. These meat pieces were placed in a sterile beaker and washed 10 times first with distilled water and then with sterile water twice. After thorough washing, these meat pieces were placed in sterile open petri plates under UV light (at 254 nm) for 30 minutes on each side and then stored at 4ºC for 24 hours in order to reduce bacterial counts (Murali *et al.,* 2012).

Effect of plant extracts on inoculated chicken meat:

The chicken meat pieces were transferred into individual stomacher® bags after 24 hours. Individual pieces were inoculated on the surface with 100µL of each bacterial suspensions of *C.jejuni*, i.e., 81176, PRCC, POCC and RECC strains in their respective bags. For a negative

control, one piece of chicken which was uninoculated was marinated with 5 mL PBS (1X). The bags containing the inoculated chicken pieces were held at room temperature for 30 minutes to allow bacterial attachment. A volume of 5 mL of each prepared marinade at the above mentioned concentrations were added to the individual bags containing the chicken pieces. The marinated chicken pieces were kept in the refrigerator at 4ºC and samples were taken at 2h, 6h and 24h. Stomacher bags containing the chicken pieces were taken out at these time points and were stomached for 2 minutes using a Stomacher® 400Circulator to drive bacteria into suspension. Bacterial cell counts in suspension were then enumerated by serial dilution and plating onto CE agar plates.

Measurement of antibacterial activity:

All the plates were incubated at 42ºC under microaerophilic conditions for 48 hours after which the plates were read using a colony counter and viable cell counts recorded.

Statistical Analysis

Both experiments to study the effect of plant extracts against *C. jejuni* in broth culture as well as on chicken meat were repeated three times to establish statistical significance. Statistical analysis was performed using JMP 11.0 provided by the University of Arkansas, Fayetteville.

RESULTS

The effectiveness of commercially available plant extracts was investigated in this study against four *C. jejuni* strains including 81176, PRCC, POCC and RECC. The plant extracts selected for the analysis included green tea, oregano, hawthorn and curcumin. The initial set of experiments on the four isolates was done in broth culture at three concentration levels of 0.25%, 0.5% and

1% of the purchased extract concentration. Of the four extracts, green tea (Figure 3.1 – Figure 3.4) and oregano (Figure 3.5 – Figure 3.8) showed 7-log reduction and killing all the bacteria within 24 hours of incubation at all three concentration levels. Hawthorn showed no significant log reduction ($P>0.05$) with respect to control after 24 hours of incubation for all concentrations and isolates tested (Fig 3.9 – Fig 3.12). Curcumin at the highest concentration of 1% of the purchased extract concentration produced a minimum of 2-log reduction from the control, at 24 hour time point for all isolates of *C. jejuni* (Fig 3.13 – Fig 3.16).

In order to narrow down the effective concentrations of the extracts in the meat model study, 81176 and PRCC isolates were selected to represent the human strain and poultry strain. Concentrations of 1%, 5%, 10% and 20% of the purchased extract concentration were tested at time points of 2 hour and 24 hour to narrow down the range of effective extract concentrations (Table 3.1). Based on these initial results, chicken breast pieces then were inoculated with the 81176, PRCC, POCC and RECC isolates and were marinated with 50% and 100% of the purchased concentrations of green tea extract, 30% and 50% of the purchased concentrations of oregano extract and 50% and 100% of the purchased concentration of hawthorn extract. Marinades prepared using either 50% of the purchased hawthorn extract, 100% of the purchased hawthorn extract, 50% of the purchased green tea extract or 100% of the purchased green tea extract did not kill all the bacteria within 24 hours for all four strains of *C.jejuni*. Oregano at concentration 50% of the purchased extract concentration showed 6-log reduction of 81176, PRCC, POCC and RECC *C. jejuni* strains. Oregano at 30% of the purchased extract concentration showed 6-log reduction against POCC and RECC compared to 2-log reduction against 81176 and PRCC strains (Fig 3.17 – Fig 3.20).

DISCUSSION

The effect of tea extract on *C. jejuni* in broth was reported as killing all the bacteria in 4 hours (Diker, *et al*., 1991). No reference is given for the bacterial strains used or the concentration of the extract which makes it difficult to compare results. In our study, green tea was successful in killing all the *C.jejuni* in broth in 24 hours which is greater than reported by Murali *et al.,* (2012) on 81176 and RECC strains where the bacteria were killed within 36 hours of incubation. In the previous work of Murali *et al.,* (2012), tea extracts were prepared using the method of (Diker *et al*., 1991). The concentration of extract obtained by this method is dependent on the source and variety of the tea leaves. In our study, extracts were purchased commercially from gaia® herbs which state the extracts have "a consistent, measurable concentration of a recognized phyto-constituent" ("Beyond standardization," 2014) to account for the variability of plant product. Initial testing in the meat model, at the highest concentration used in broth study (1% of the purchased extract concentration), did not show any reduction in viable counts which required testing at higher concentrations all the way up to full concentration of the commercial extract. At 100% of the purchased concentration of green tea in meat model, a 2- log reduction in bacteria was observed at the 2 hour time point and did not decrease any further till 24 hour time point. The growth inhibition of tea polyphenols against *B.stearothermophilus* (Sakanaka *et al.*, 2000) shows a similar steady antibacterial activity at 20 hr time point for different concentrations of tea polyphenols/ml *in vitro*.

Oregano extracts, in broth culture study, demonstrated a 7-log reduction in bacterial counts for all strains of *C.jejuni* after 24 hours. Thymol and carvacol, two major components of oregano have shown < 1 Log CFU values at concentrations of 10, 20 & 30 m*M* by 8 hours of incubation and maintained <1 Log CFU by 24 hours from 4.5 Log CFU values at time 0 hour (Kollannor *et al.,* 2010). This trend matches the results observed in the 0.25%, 0.5% and 1.0% of the purchased

concentrations of the extracts tested. When testing in the meat model, the minimum concentration of oregano that showed reduction in bacterial count was 30% of the purchased concentration in the POCC and RECC isolates of *C. jejuni*. The bacteria were completely killed using the 50% of the purchased concentration in all *C. jejuni* strains indicating that the 30% of the purchased concentration of oregano is very close to the minimum inhibitory concentration of the extract in the meat model. Testing carried out to determine the effect of oregano origanum oils on RM1221, RM1230, RM1274 & RM1046 strains of *C.jejuni* revealed increased sensitivity to essential oils (Friedman *et al.*, 2002). The results in the *in vitro* study by Friedman *et al.*, 2002, do not show variation of oregano oil antibacterial activity between the different *C.jejuni* strains. This is very similar to the results observed in our broth culture study. In our meat model study differences were observed in the antibacterial action of oregano on POCC (post chilled) and RECC (retail chicken carcass) isolates as compared to 81176 (human strain) and PRCC (pre-chilled) isolates. One possible explanation is the effect of chilling on the *C.jejuni* pathogen after exposure to low temperatures affecting the antibacterial action of oregano on them. A difference in rate of death in certain strains of *C.jejuni* was observed after exposure of culture to 6°C for 24 hours (Hughes *et al*., 2010).

Hawthorn extract did not exhibit log reduction similar to that observed in green tea and oregano extracts in broth culture against all four isolates of *C.jejuni* through the 24 hours of incubation. In meat model tests the hawthorn extract at 100% of the purchased concentration exhibited a 1-log reduction at the 24 hour time point. This result is in agreement with experiments using extracts of *Crataegus monogyna* commonly known as hawthorn where less than 25% growth inhibition was observed with *C.jejuni* (Galgóczy *et al.*, 2009). Unfortunately no reference is made in the study to the concentration of the extract or the isolate of *C.jejuni* used.

Curcumin experiments were conducted only in broth culture to see the effect of the extract at low concentrations. At the 24 hour time point a minimum of 2 log-reduction was observed for all strains of *C.jejuni* at 1% concentration. The broth culture studies of curcumin were primarily part of the study to determine suitable extracts to test on meat model. The initial tests in meat model indicated the requirement of higher concentrations for eliminating bacteria at 24 hour time point for all strains and the cost of curcumin was higher than other extracts which limited the range of testing.

There were major differences in concentrations of extracts required to inhibit the bacteria between broth medium tests and meat model tests. This has been recognized as a potential drawback of using plant extracts in the food (Davidson *et* al., 2013; Hayek *et al*., 2013; Škrinjar *et al*., 2009). This difference may be due to interaction of plant extracts with components present in food model such as high fat and protein content (Burt, 2004), lipids (Perumalla *et al*., 2011) and water activity. According to Hayek *et* al., 2013, pH levels in meat may influence the hydrophobic effect of extracts and in turn their antibacterial effect.

CONCLUSIONS

Future tests will involve isolating individual components of each of the extracts determining the dominant antibacterial component with minimal inhibitory concentrations (MIC). The higher concentration requirements of plant extracts in meat models can be resolved by determining combinations of lower concentration plant extracts that can inhibit bacterial growth and avoid undesirable sensory changes (Hayek *et* al., 2013).

FIGURE LEGEND

Fig 3.1. Log CFU vs Time of green tea extract at 3 different concentrations against *C.jejuni* 81176. Green tea was effective at killing bacteria within 24 hours of incubation. There was a 7-log decrease in growth in 24 hours for all 3 concentration levels ($P < 0.05$).

Fig 3.2. Log CFU vs Time of green tea extract at 3 different concentrations against *C.jejuni* PRCC. For all 3 concentration levels, green tea showed a 7-log reduction from the control in 24 hours. Although green tea at 1% concentration showed a 1-log decrease in growth in 4 hours, it killed all the bacteria in 24 hours ($P < 0.05$).

Fig 3.3. Log CFU vs Time of green tea extract at 3 different concentrations against *C.jejuni* POCC. Green tea showed a 7- log reduction from the control in 24 hours ($P < 0.05$). There was no significant difference in the action of 0.25% and 0.5% concentrations against the isolate.

Fig 3.4. Log CFU vs Time of green tea extract at 3 different concentrations against *C.jejuni* RECC. All three concentrations of green tea showed a 7-log reduction from the control in 24 hours ($P < 0.05$). Green tea at 1% showed a 1-log reduction in 4 hours, but there was a steady decline in count after that.

Fig 3.5. Log CFU vs Time of oregano extract at 3 different concentrations against *C.jejuni* 81176. Oregano was effective at killing bacteria within 24 hours, showing a 7- log reduction ($P<0.05$). Although oregano 1% showed a 1- log reduction in 4 hours, there was a steep decline in growth after that.

Fig 3.6. Log CFU vs Time of oregano extract at 3 different concentrations against *C.jejuni* PRCC. All concentrations of oregano were effective in killing bacteria within 24 hours ($P < 0.05$).

Fig 3.7. Log CFU vs Time of oregano extract at 3 different concentrations against *C.jejuni* POCC. For all 3 concentrations of oregano, there was an 8- log decrease ($P < 0.05$) killing all the bacteria in 24 hours. Oregano 1% showed a 1- log decrease in 4 hours with a steady decline after that.

Fig 3.8. Log CFU vs Time of oregano extract at 3 different concentrations against *C.jejuni* RECC. Oregano was found to be effective in killing bacteria within 24 hours of incubation ($P<0.05$). There was a 1- log decrease for oregano 1% in 4 hours of incubation.

Fig 3.9. Log CFU vs Time of hawthorn extract at 3 different concentrations against *C.jejuni* 81176. All the 3 concentrations did not show significant difference in their action against the isolate for all time points. Also, hawthorn at all concentrations failed to kill the bacteria within 24 hours of incubation.

Fig 3.10. . Log CFU vs Time of hawthorn extract at 3 different concentrations against *C.jejuni* PRCC. Hawthorn 1% showed more than 1- log reduction in growth at 24 hours.

Fig 3.11. Log CFU vs Time of hawthorn extract at 3 different concentrations against *C.jejuni* POCC. Hawthorn 1% showed a 1- log difference in 24 hours. There was no significant difference observed between the concentrations at 24 hour time point.

Fig 3.12. Log CFU vs Time of hawthorn extract at 3 different concentrations against *C.jejuni* RECC. Although a slight decrease was seen from the control in 24 hours for all the three concentrations, there was no significant difference in its action between the concentrations against the isolate.

Fig 3.13. Log CFU vs Time of curcumin extract at 3 different concentrations against *C.jejuni* 81176. Curcumin 1% showed more than 2- log reduction in 24 hours whereas 0.25% and 0.5% showed less than 1- log reduction with no significant difference in its action between the two concentrations.

Fig 3.14. Log CFU vs Time of curcumin extract at 3 different concentrations against *C.jejuni* PRCC. Curcumin 1% was found to be effective among the three concentrations and showed a 3-log reduction in 24 hours of incubation whereas 0.25% and 0.5% concentrations showed a 2 – log decrease in growth.

Fig 3.15. Log CFU vs Time of curcumin extract at 3 different concentrations against *C.jejuni* POCC. Curcumin 1% showed a 2- log reduction in 24 hours of incubation.

Fig 3.16. Log CFU vs Time of curcumin extract at 3 different concentrations against *C.jejuni* RECC. Curcumin 1% showed a 3- log decrease in 24 hours of incubation. Curcumin 0.25% and 0.5% concentrations showed more than 2- log reduction, in 24 hours.

Fig 3.17. Log CFU vs Time of oregano, green tea and hawthorn extract marinated on chicken breast pieces at 2 different concentration levels against *C.jejuni* 81176. Oregano at 50% succeeded in killing all bacteria within 2 hours of incubation resulting in a 6- log reduction ($P < 0.05$) whereas oregano 30% showed a 2 – log decrease in 24 hours. Green tea 100% showed 2-log reduction whereas green tea 50% and hawthorn 100% showed a 1 – log reduction in 2 hours of incubation.

Fig 3.18. Log CFU vs Time of oregano, green tea and hawthorn extract on chicken breast pieces at 2 different concentration levels against *C.jejuni* PRCC. Oregano at 50% was the most effective of the extracts which succeeded in killing all bacteria within 2 hours of incubation ($P < 0.05$).

Fig 3.19. Log CFU vs Time of oregano, green tea and hawthorn extract on chicken breast pieces at 2 different concentrations against *C.jejuni* POCC. Oregano at 30% and 50% succeeded in killing all bacteria within 6 hours of incubation ($P < 0.05$).

Fig 3.20. Log CFU vs Time of oregano, green tea and hawthorn extract on chicken breast pieces at 2 different concentrations against *C.jejuni* RECC. Oregano at 30% and 50% succeeded in killing all bacteria within 6 hours of incubation ($P < 0.05$).

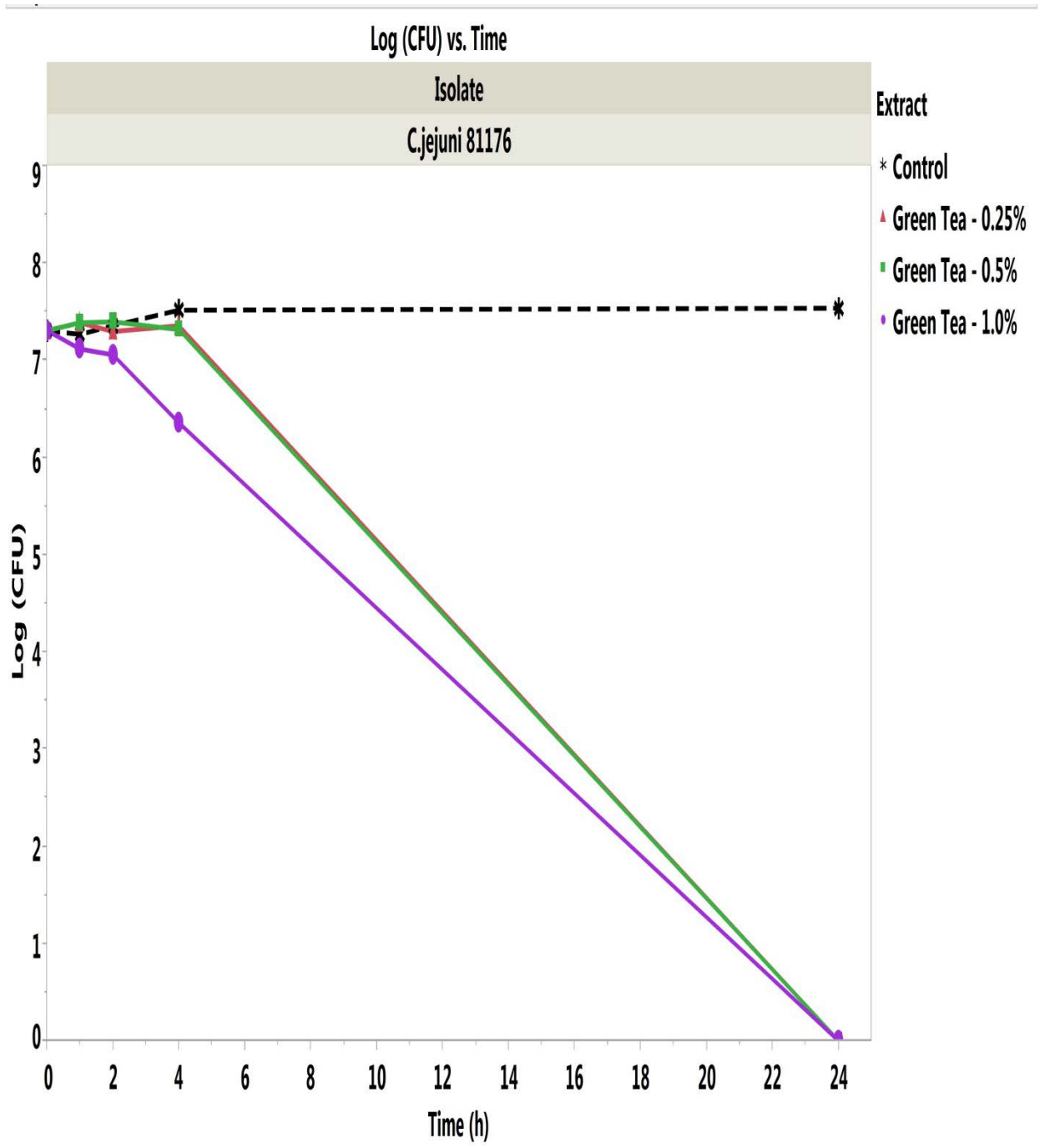


Fig 3.1. Log CFU vs Time of green tea extract at 3 different concentrations against *C.jejuni* 81176. Green tea was effective at killing bacteria within 24 hours of incubation. There was a 7-log decrease in growth in 24 hours for all 3 concentration levels ($P < 0.05$).

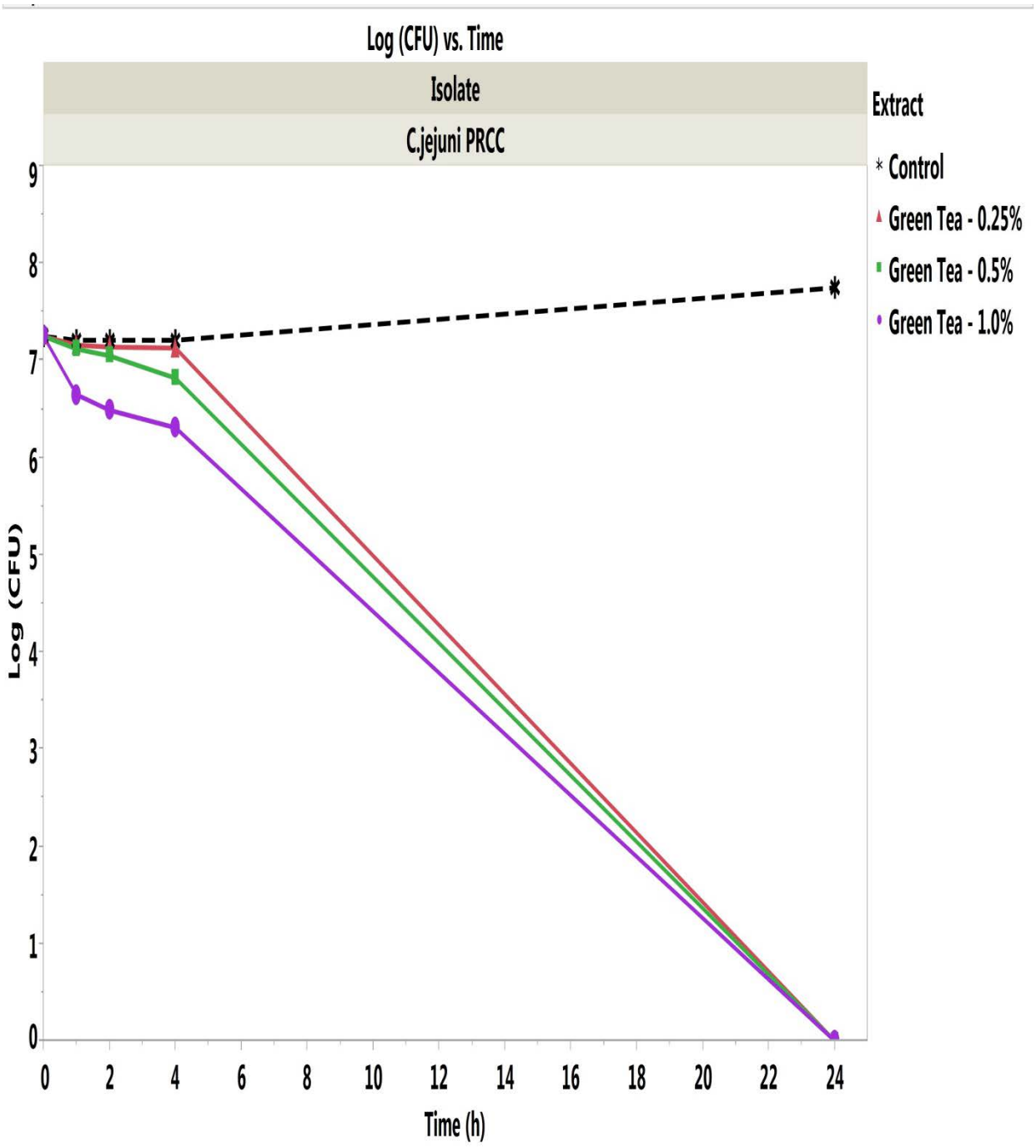
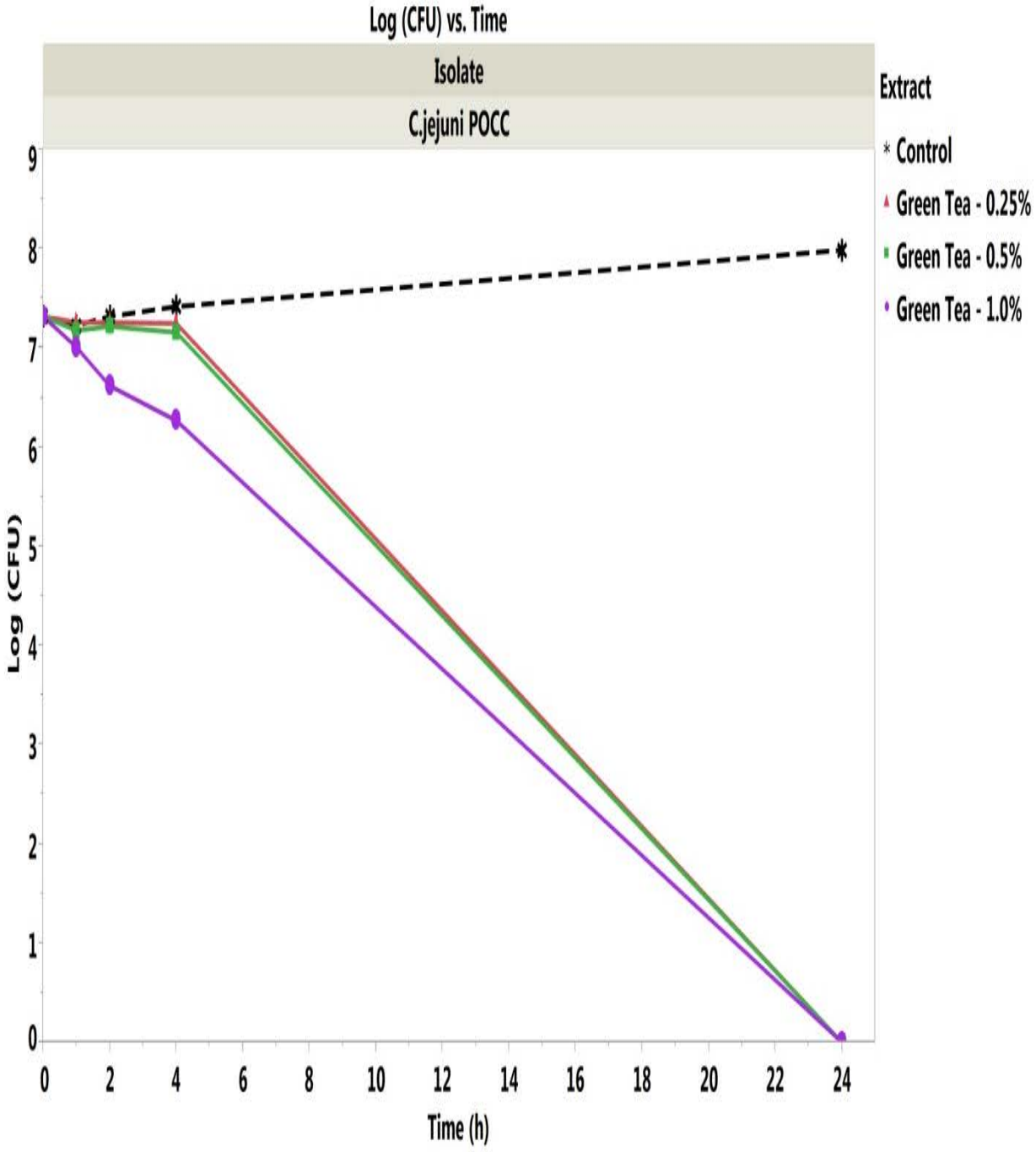


Fig 3.2. Log CFU vs Time of green tea extract at 3 different concentrations against *C.jejuni* PRCC. For all 3 concentration levels, green tea showed a 7-log reduction from the control in 24 hours. Although green tea at 1% concentration showed a 1-log decrease in growth in 4 hours, it killed all the bacteria in 24 hours ($P < 0.05$).



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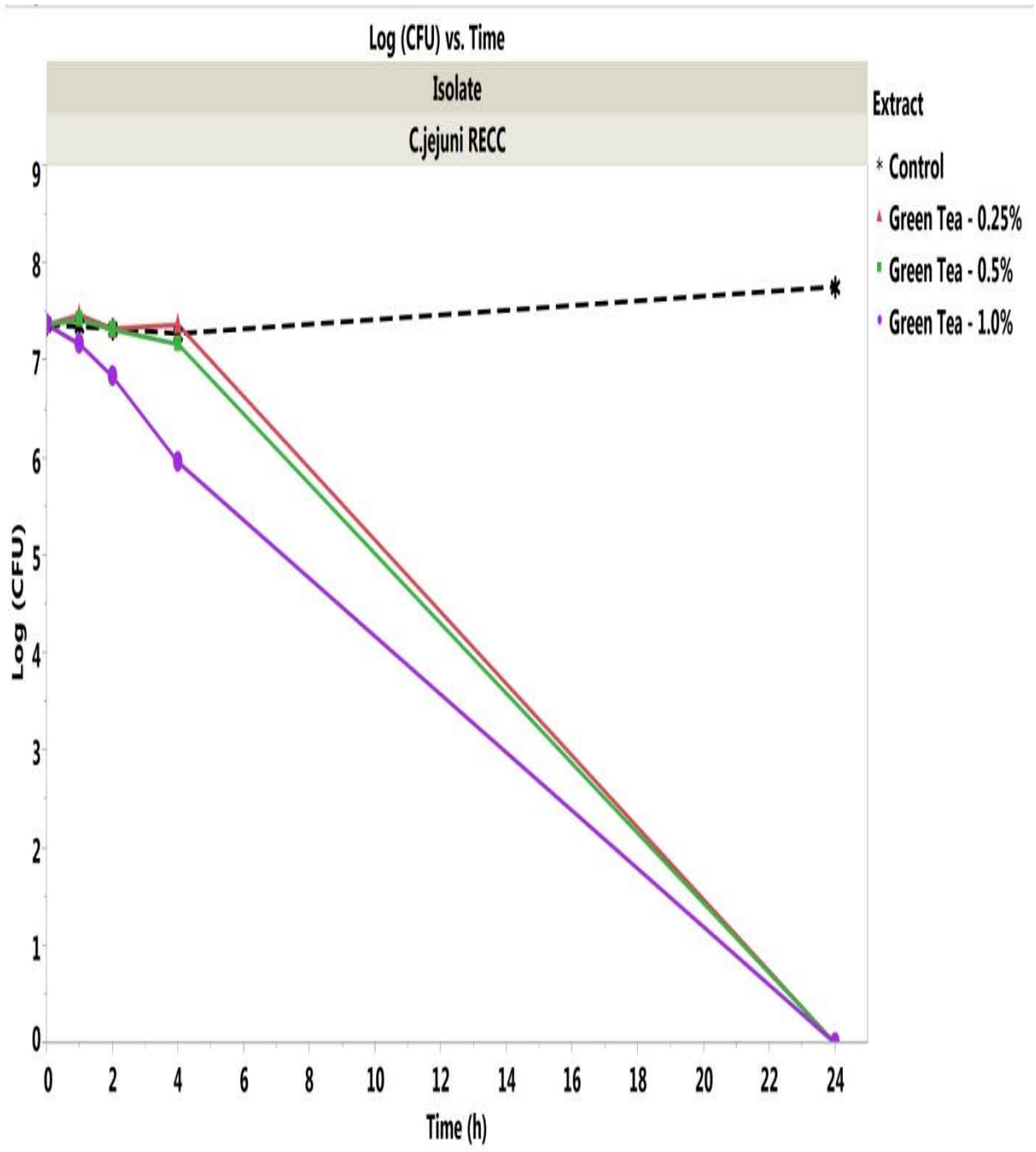

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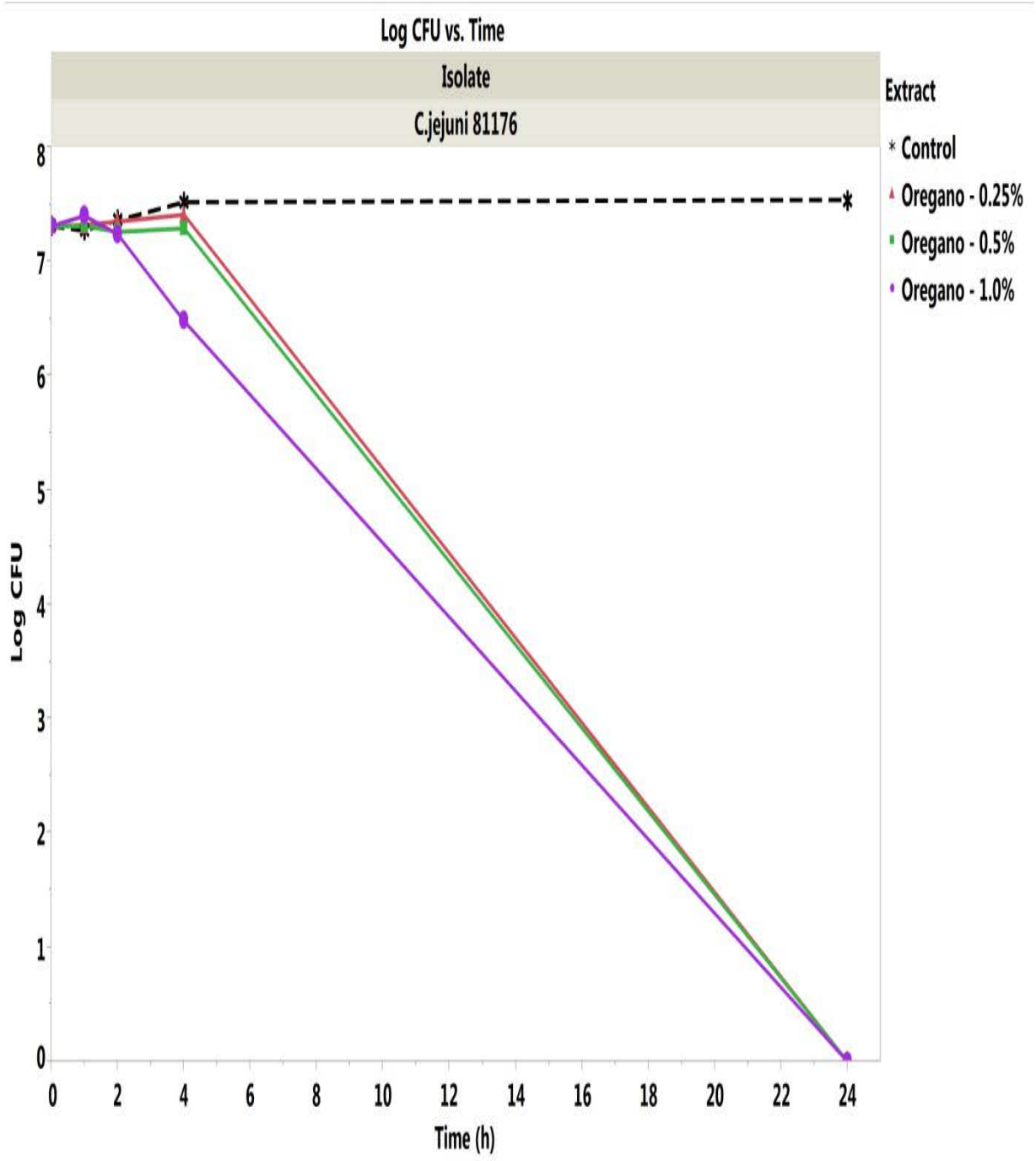
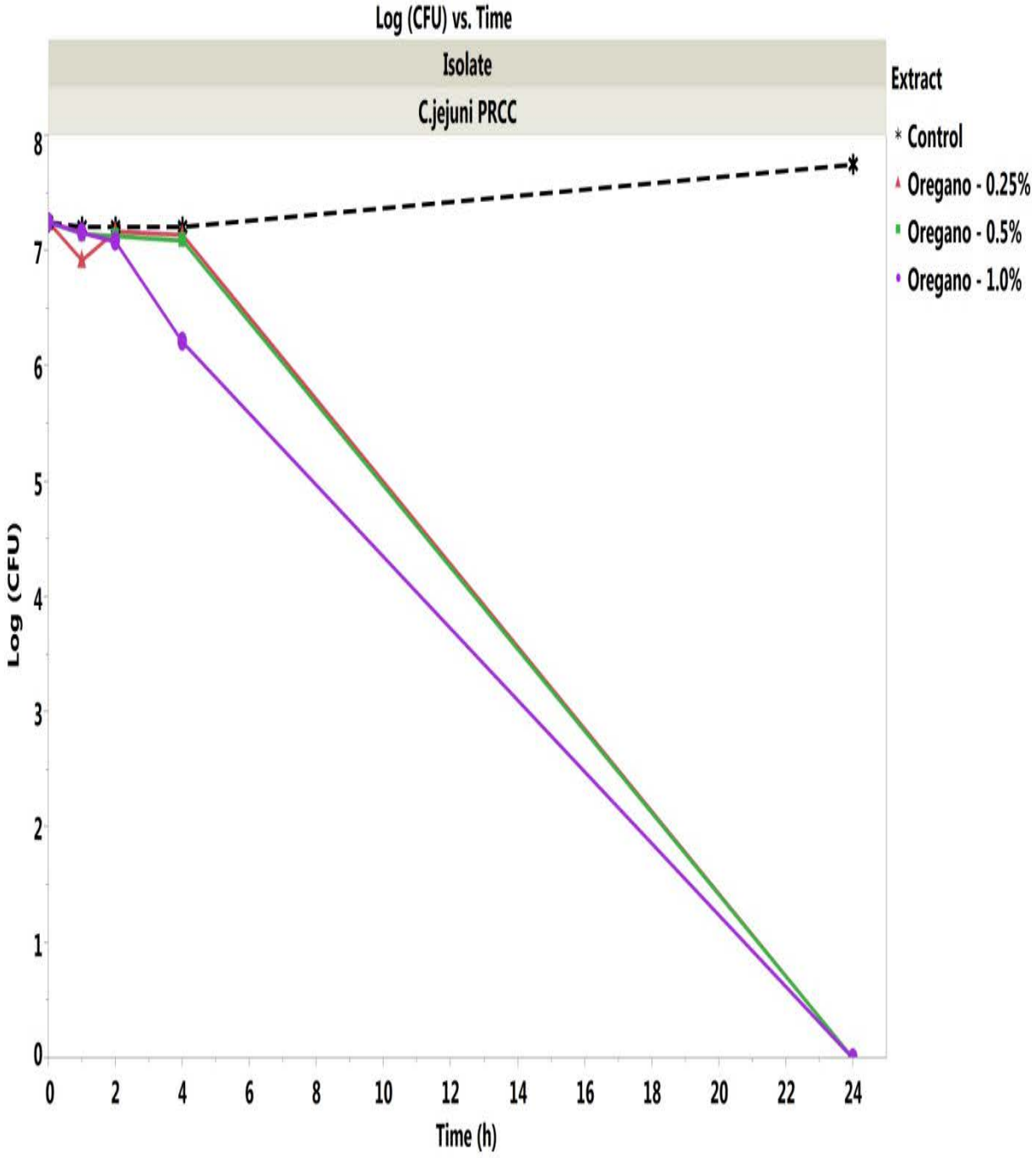


Fig 3.5. Log CFU vs Time of oregano extract at 3 different concentrations against *C.jejuni* 81176. Oregano was effective at killing bacteria within 24 hours, showing a 7- log reduction ($P<0.05$). Although oregano 1% showed a 1- log reduction in 4 hours, there was a steep decline in growth after that ($P < 0.05$).



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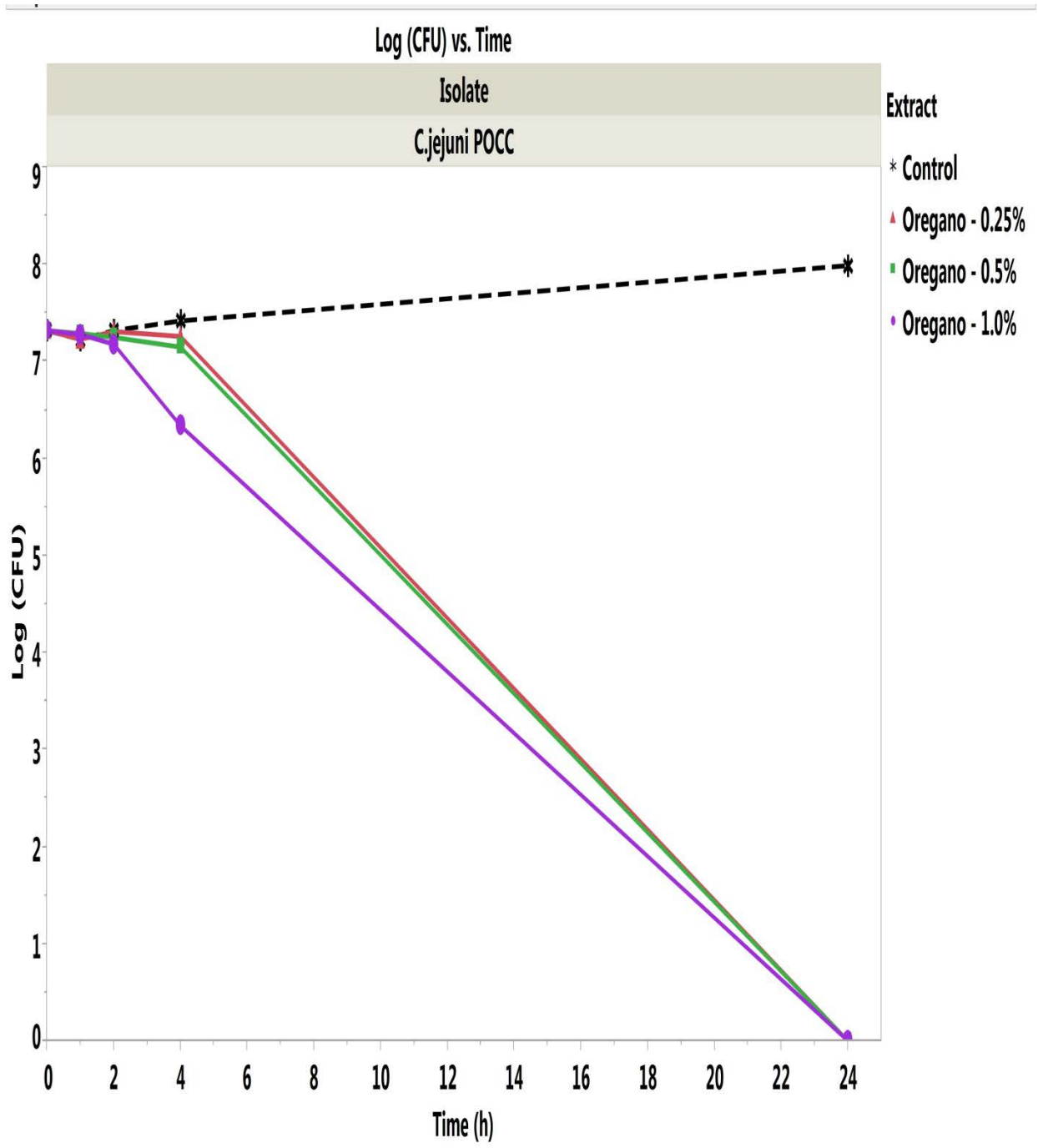

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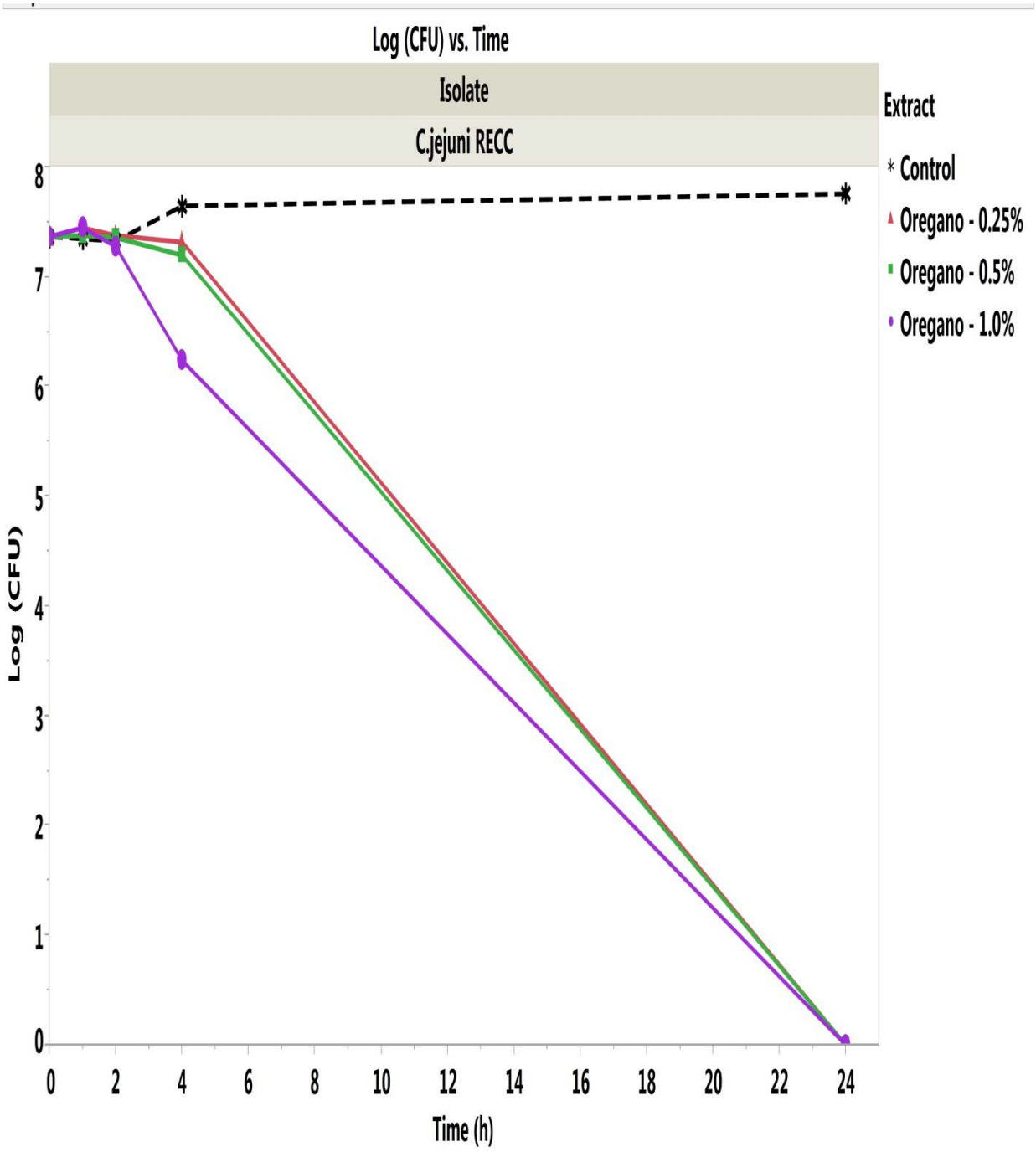

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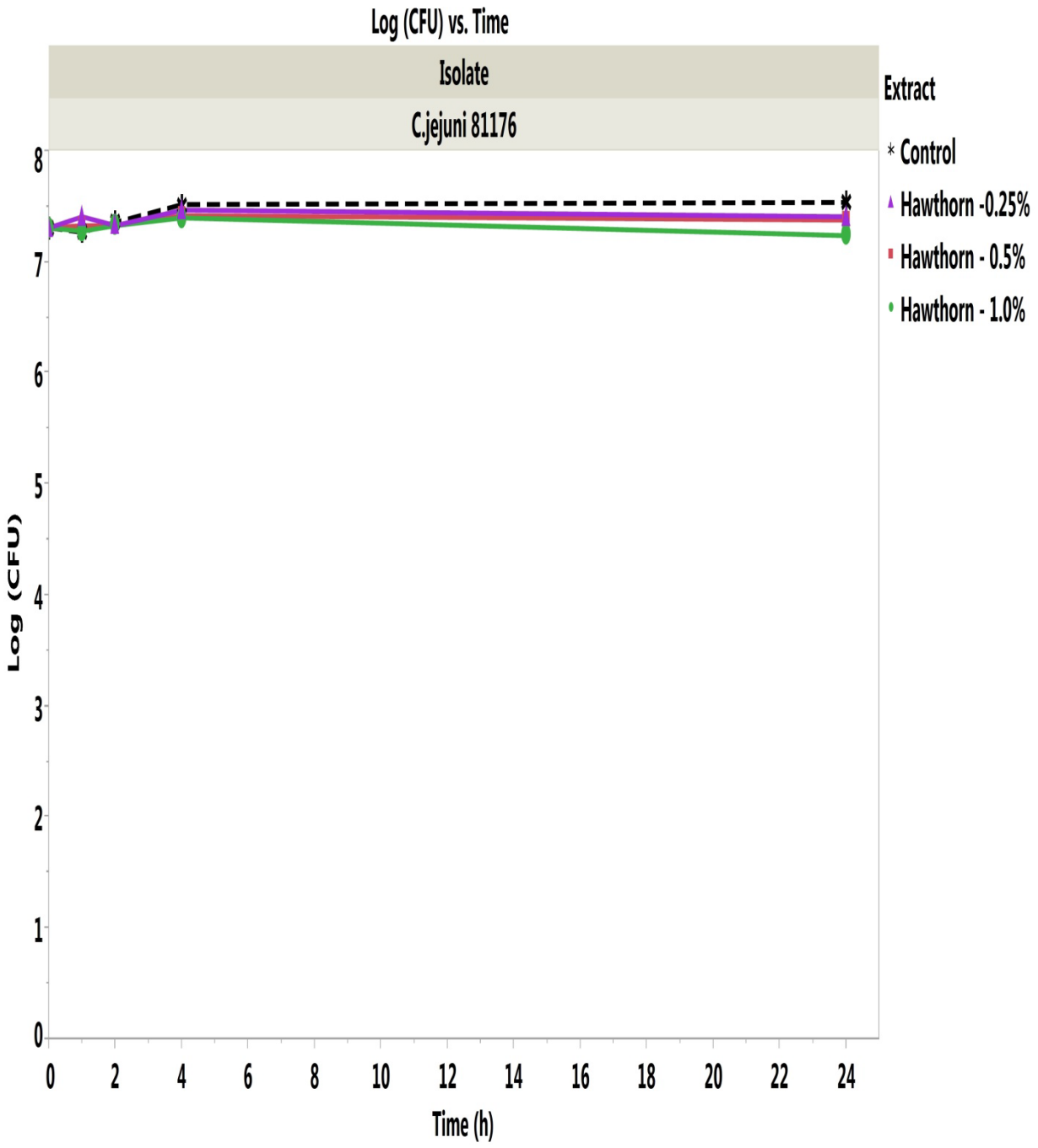

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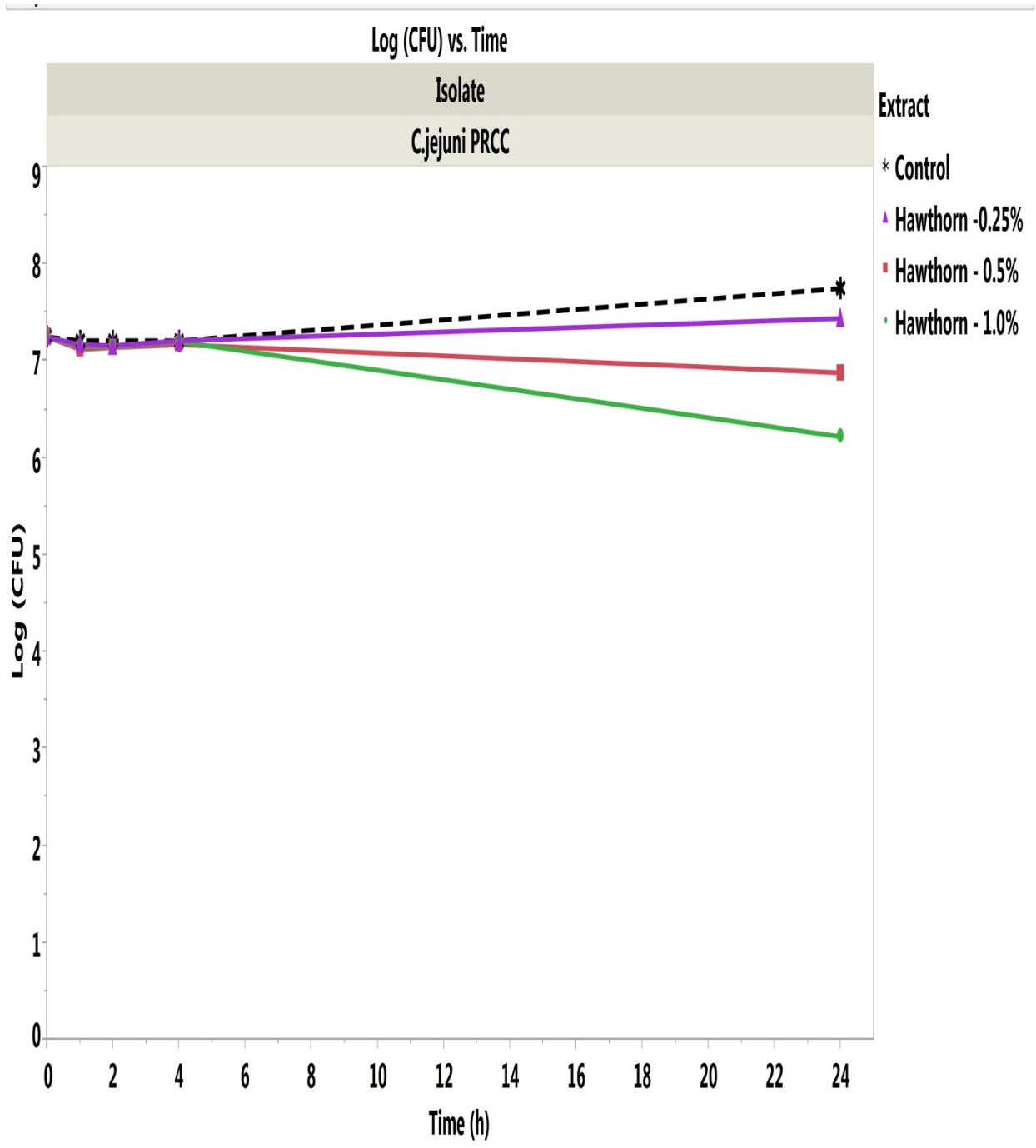

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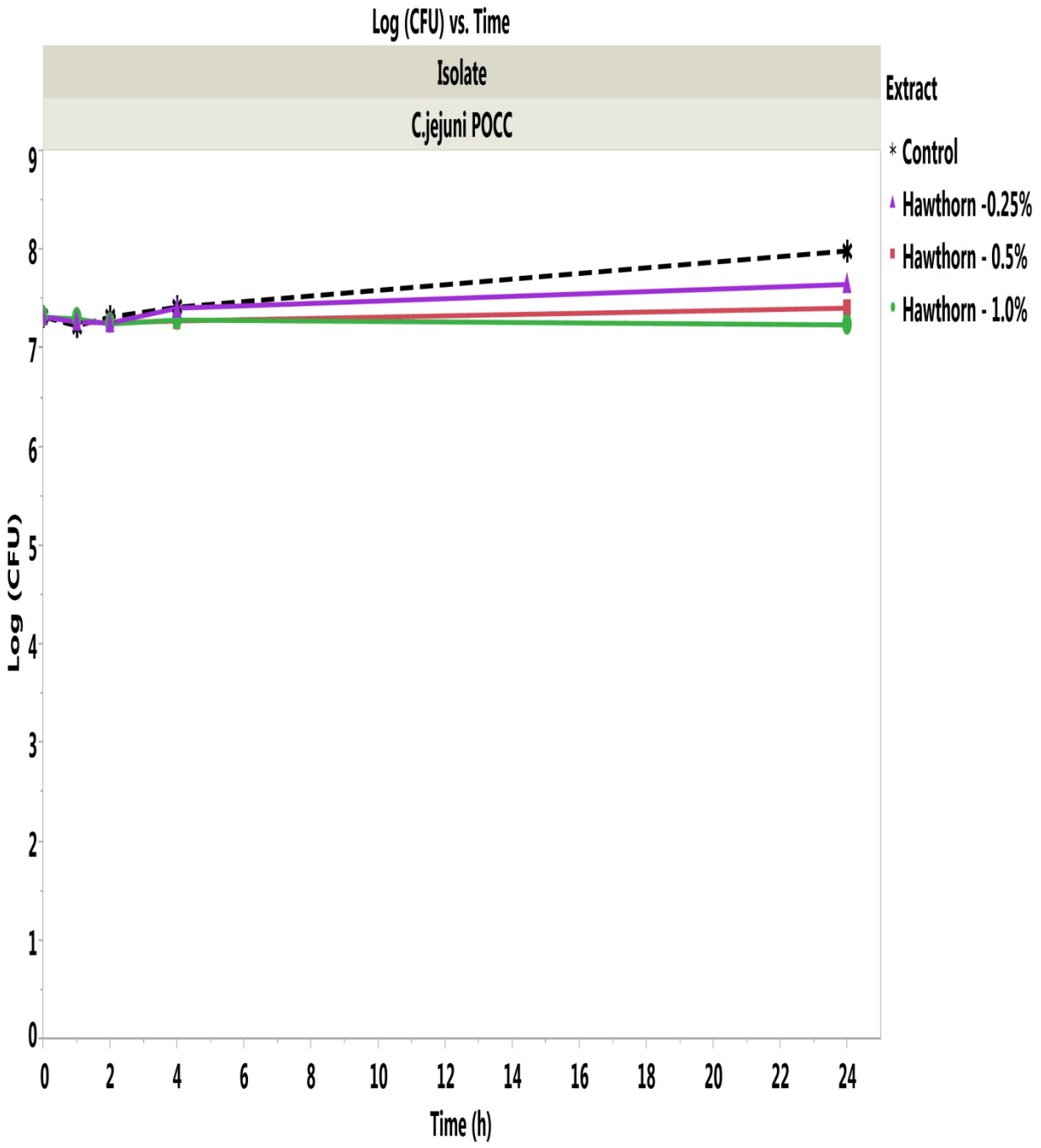

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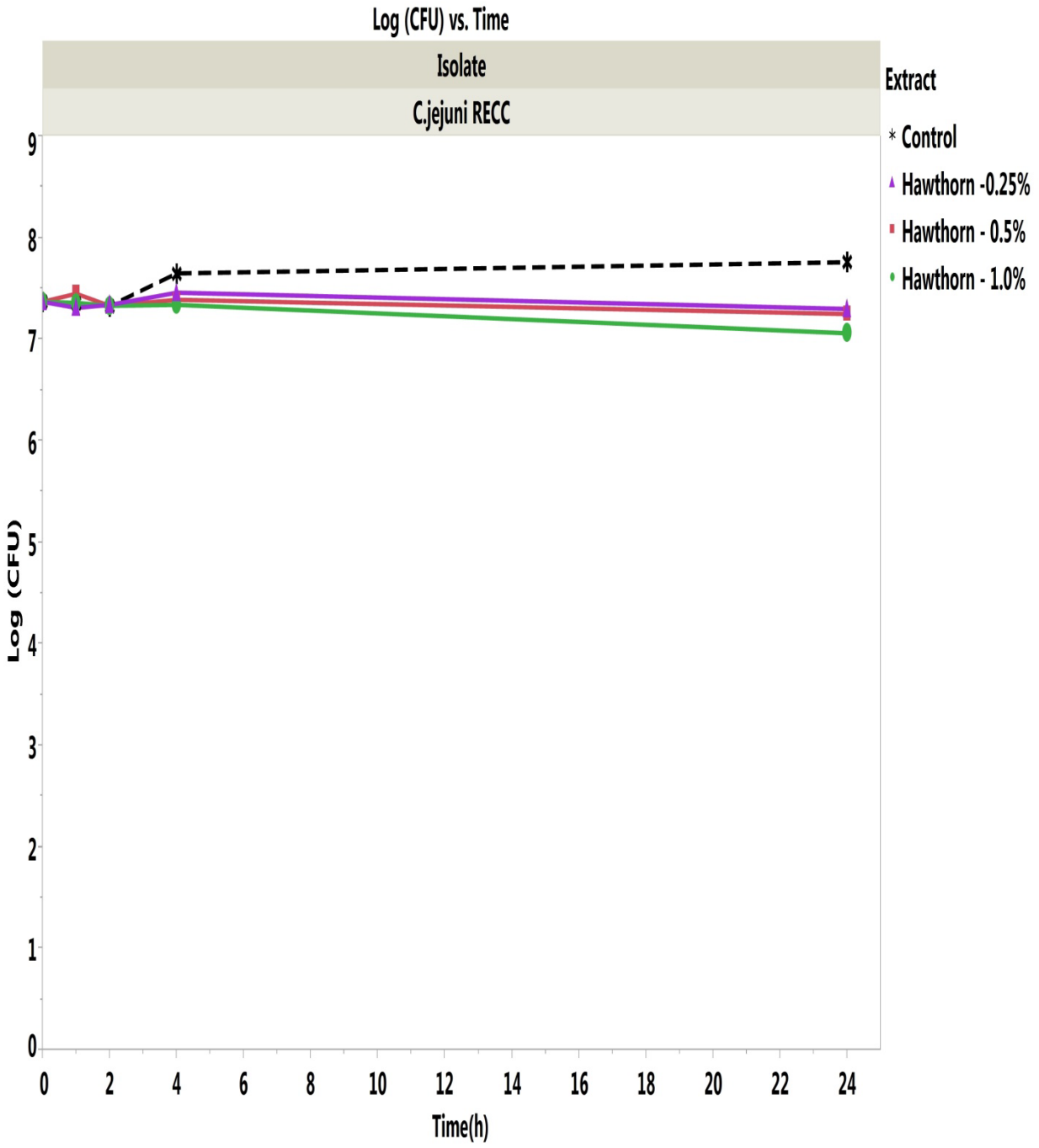

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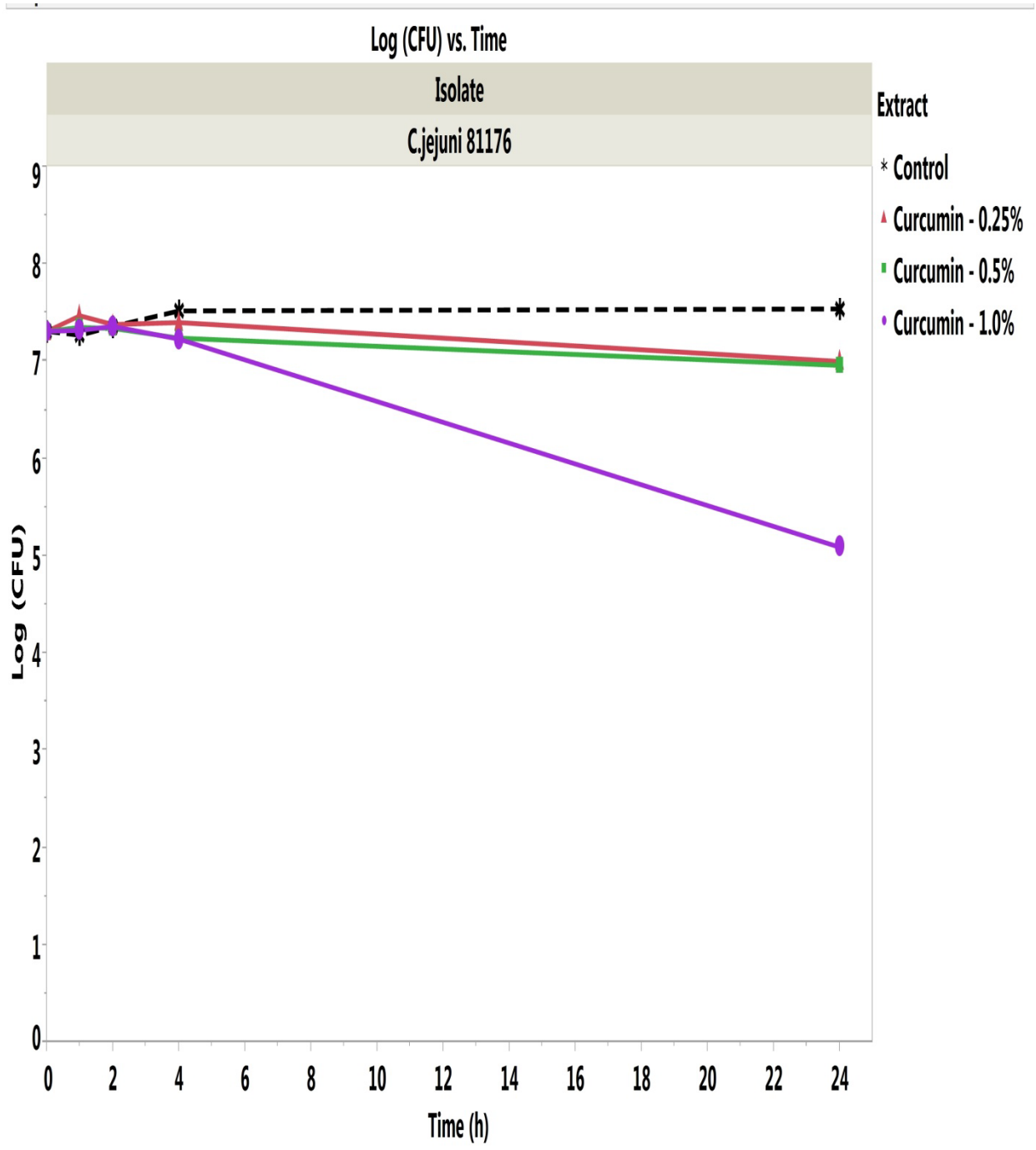

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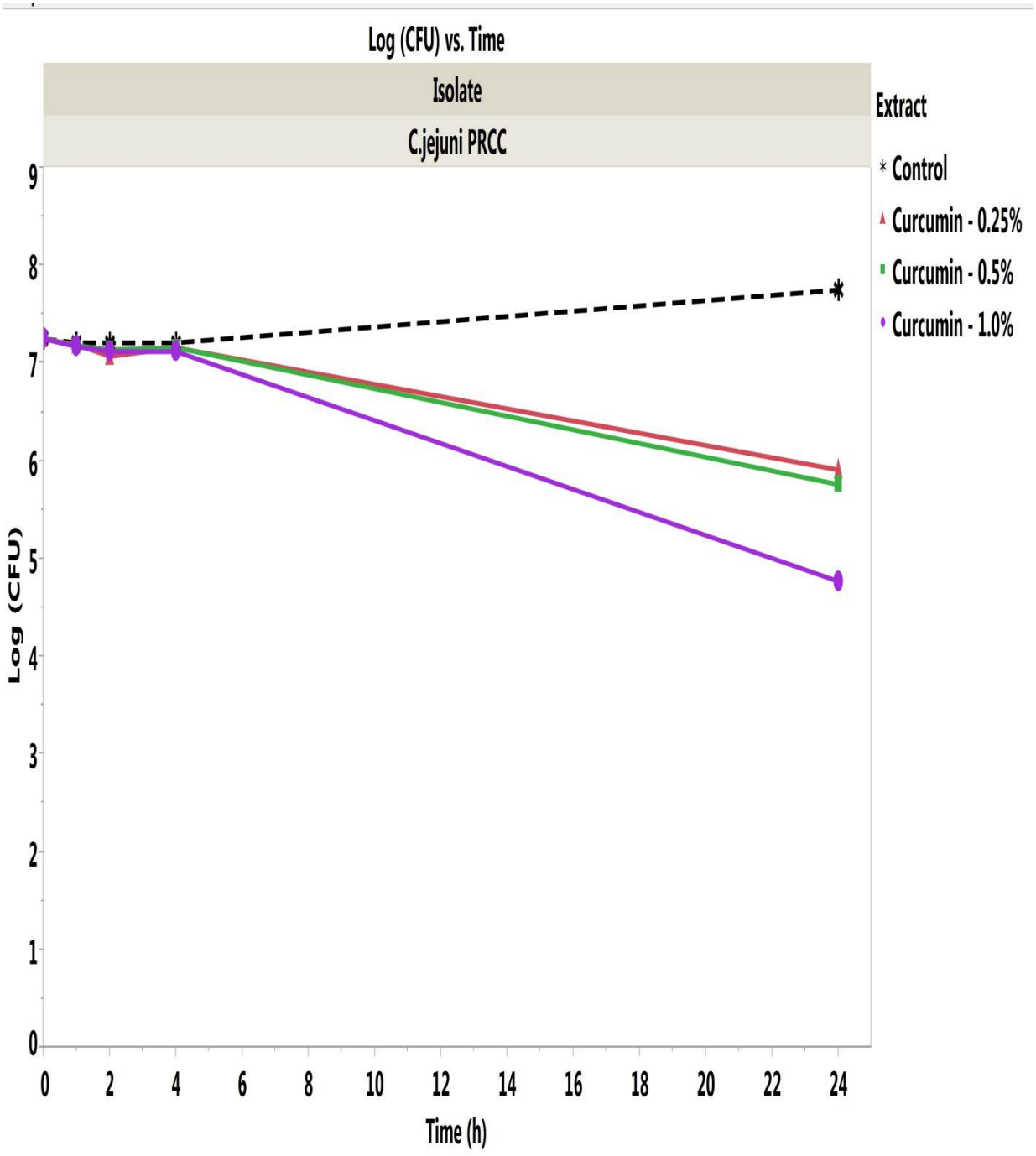

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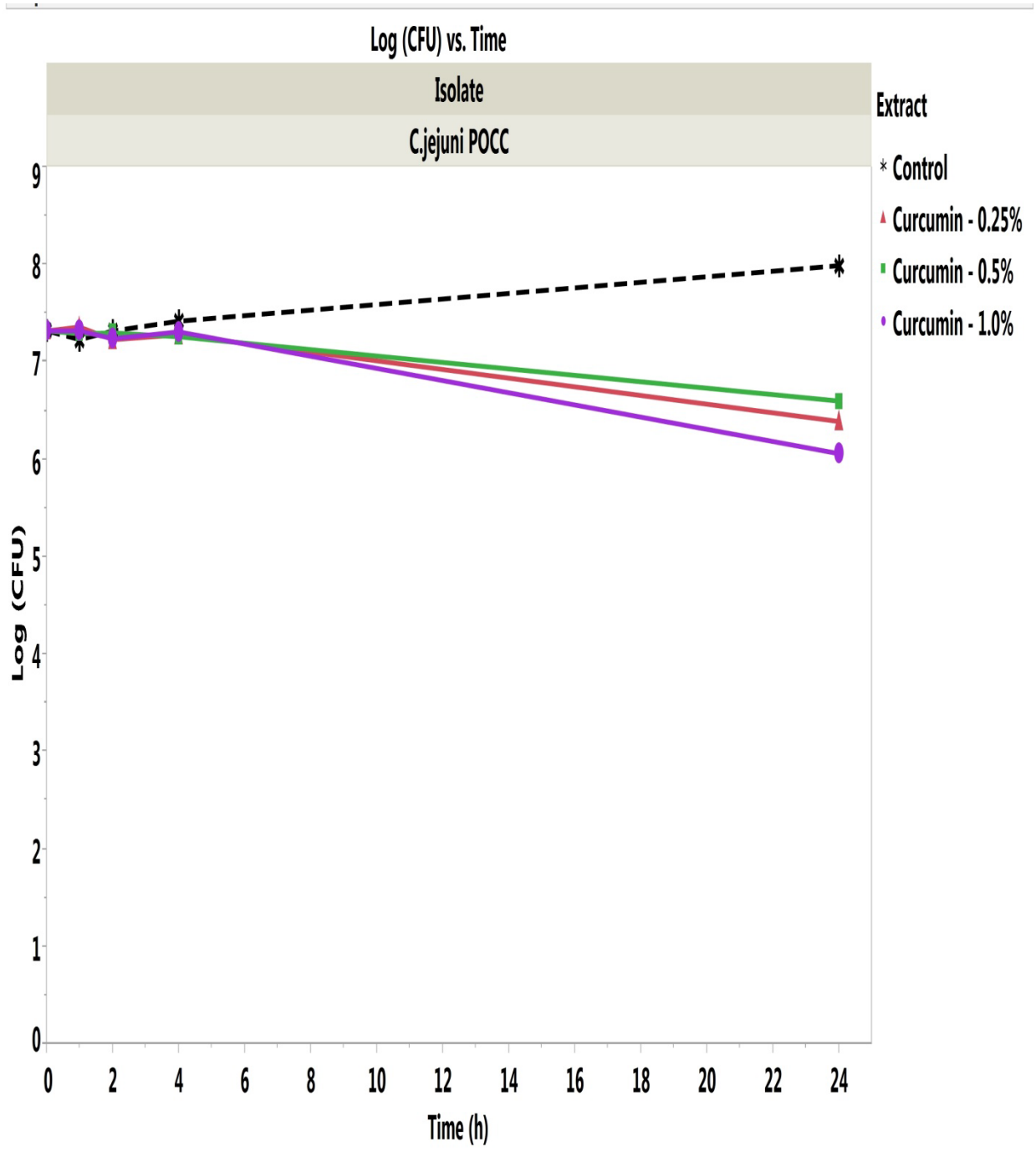

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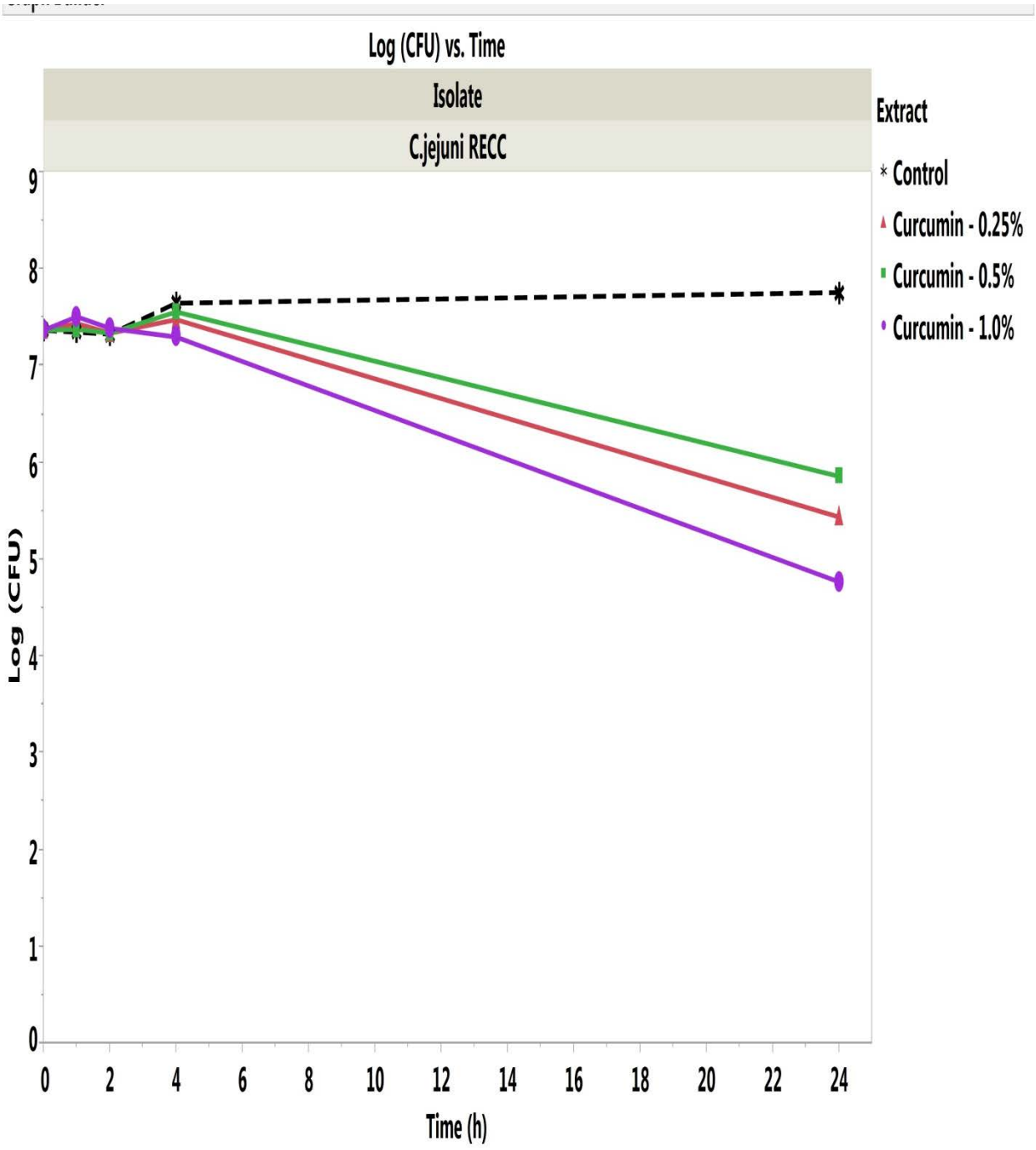

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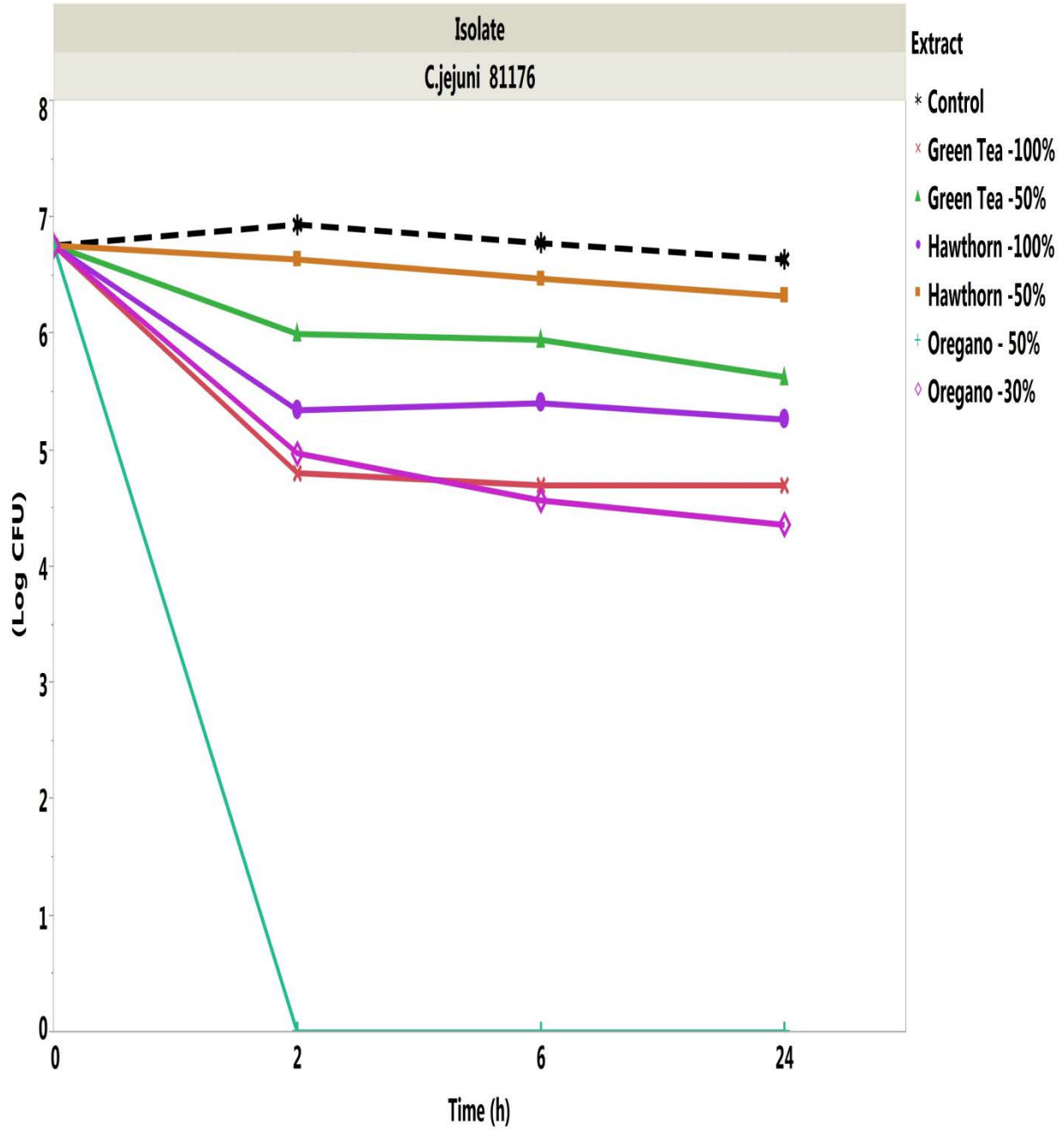
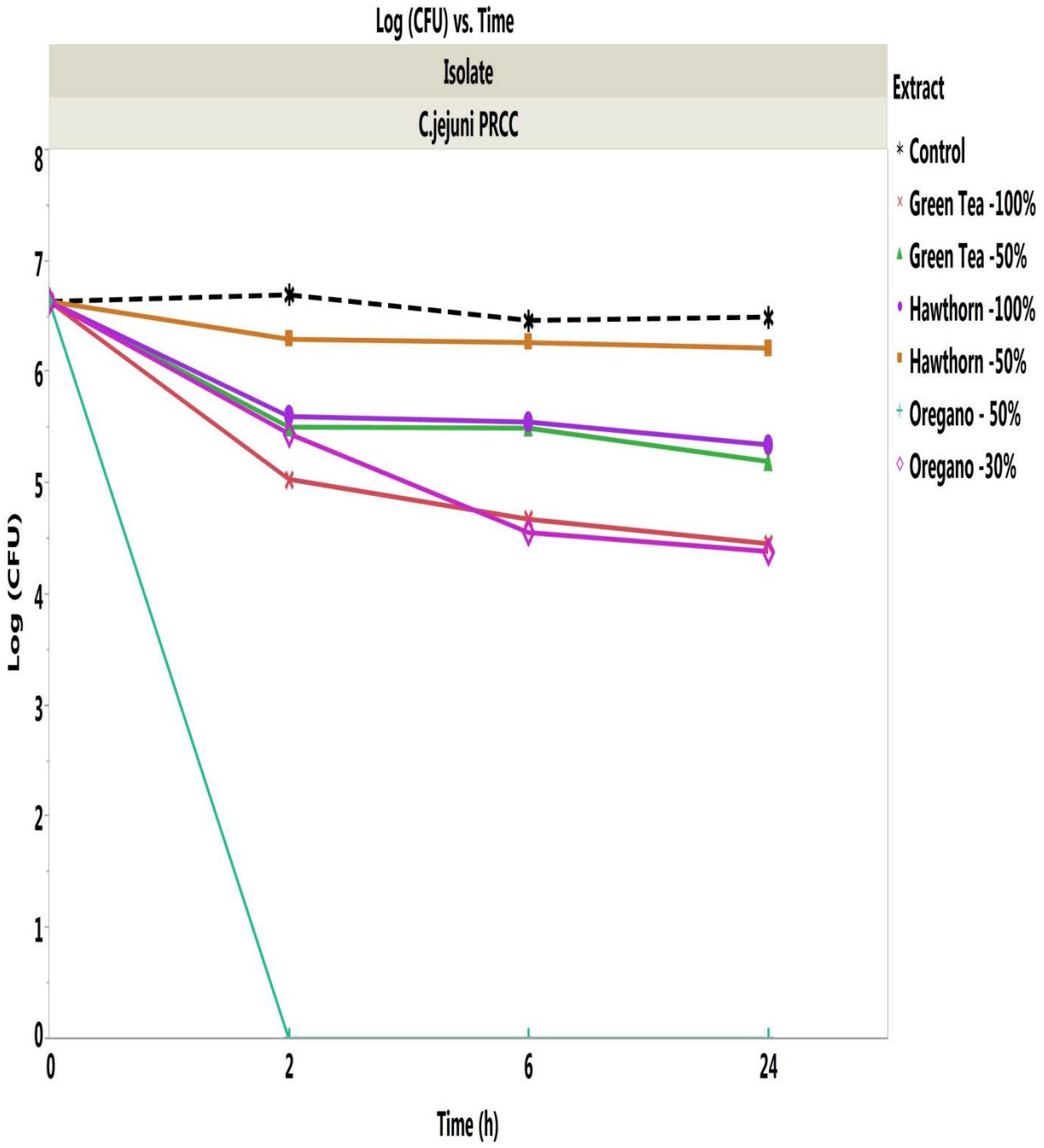


Fig 3.17. Log CFU vs Time of oregano, green tea and hawthorn extract marinated on chicken breast pieces at 2 different concentration levels against *C.jejuni* 81176. Oregano at 50% succeeded in killing all bacteria within 2 hours of incubation resulting in a 6- log reduction ($P < 0.05$) whereas oregano 30% showed a 2 – log decrease in 24 hours. Green tea 100% showed 2-log reduction whereas green tea 50% and hawthorn 100% showed a 1 – log reduction in 2 hours of incubation.



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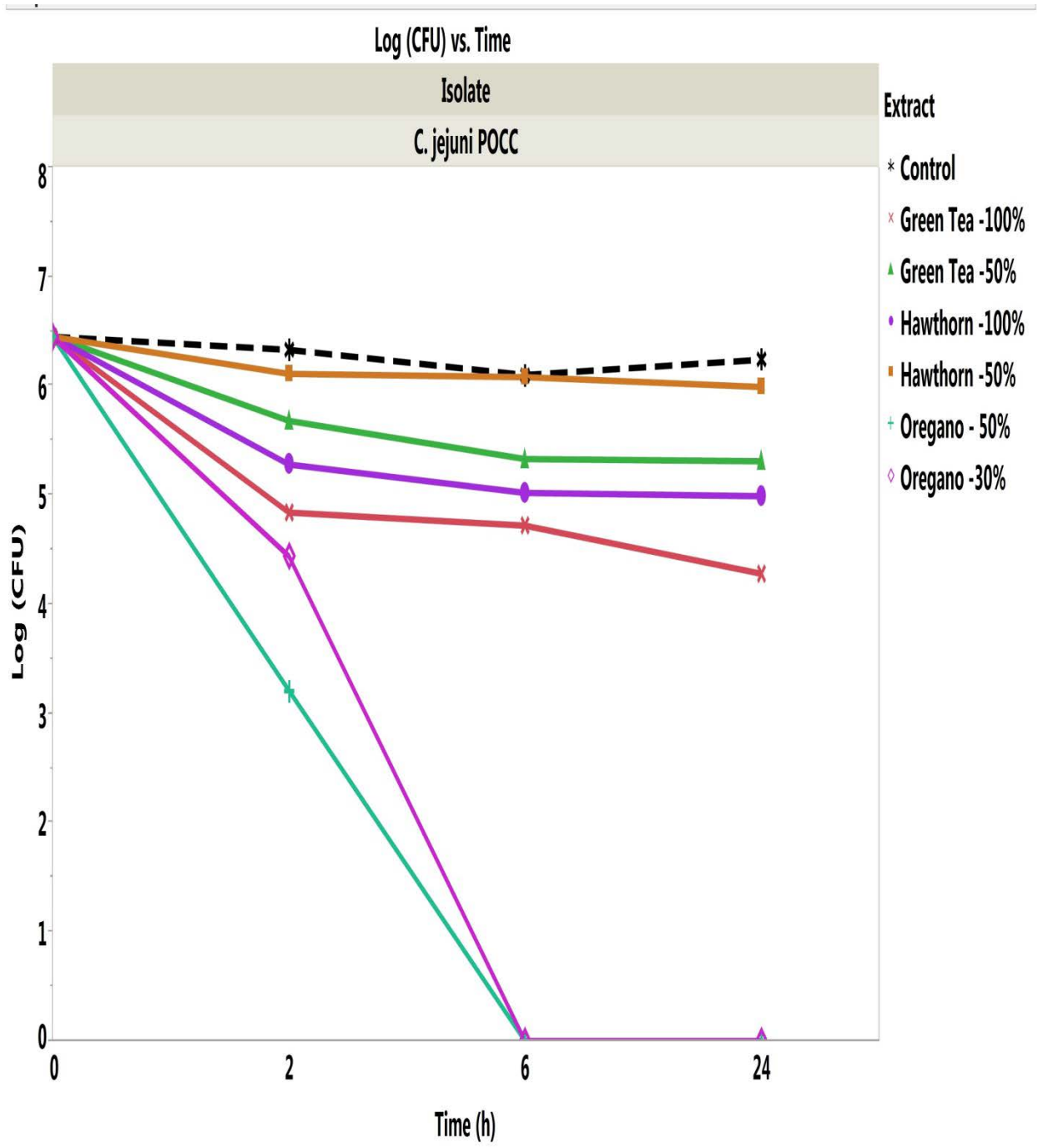

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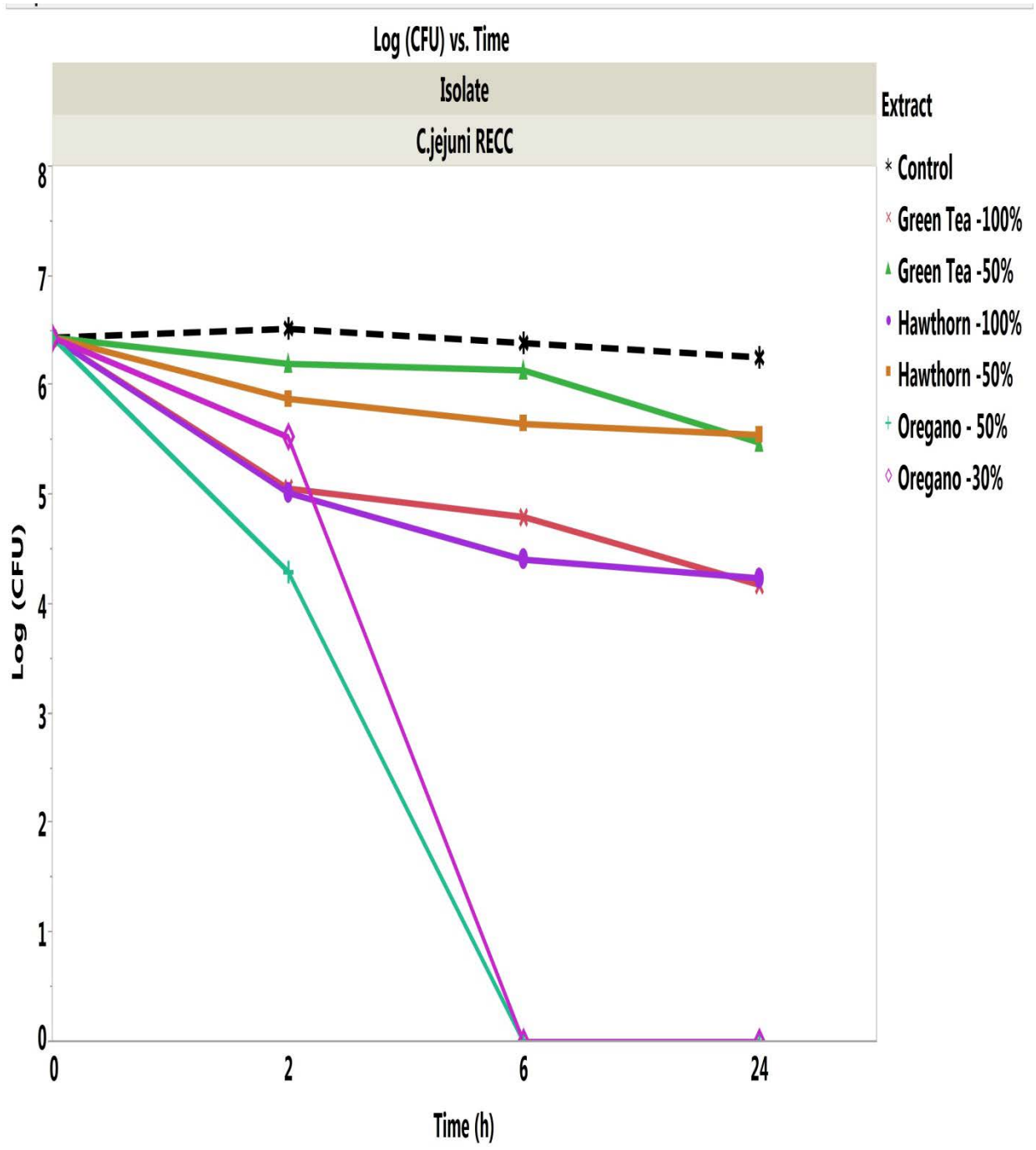

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Table 3.1. Log CFU at 2hr/24 hr time point for Oregano and Green Tea to determine concentrations that reduced bacteria over a 2 hour period in chicken breast fillet compared to in vitro studies. The maximum concentration that effectively killed bacteria within 24 hours in all strains tested was the starting point for meat model study.

PLANT EXTRACTS	***C.jejuni* 81176 - Log CFU**		***C.jejuni* PRCC - Log CFU**	
	2 hour	**24 hour**	**2 hour**	**24 hour**
Control	7	7	7	7
Green Tea - 1%	6	5	6	6
Green Tea - 5%	7	7	7	7
Green Tea - 10%	7	7	7	7
Green Tea - 20%	7	7	7	7
Oregano - 1%	6	5	6	5
Oregano - 5%	7	7	7	7
Oregano - 10%	7	7	7	7
Oregano - 20%	7	7	6	7

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CHAPTER 4

CONCLUSION

The overall aim of this study was to determine the antibacterial activity of commercially available plant extracts on selected strains of *C. jejuni*. The study involved testing these extracts against the strains of bacteria initially in broth culture and then in chicken meat model. It was found that higher concentrations were required to kill the bacteria in meat as compared to broth study. Future studies would involve isolating individual components of each of the extracts determining the dominant antibacterial component with minimal inhibitory concentrations. Combinations of plant extracts have to be explored to resolve the problem of high concentration required to inhibit *C. jejuni* strains in meat.

APPENDIX A1

EFFECT OF CURCUMIN MARINADE IN MEAT

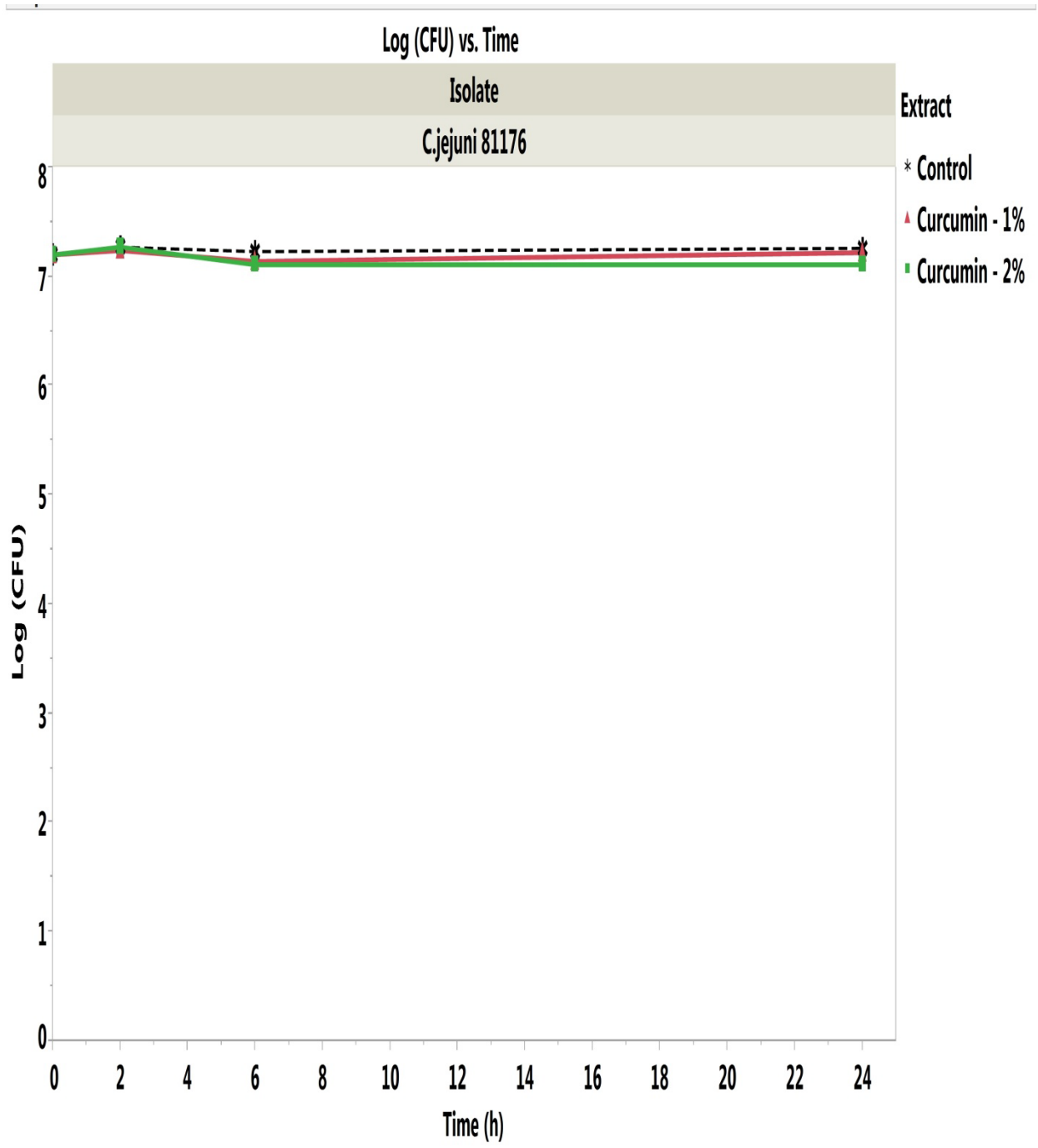

Figure A1.1. Log CFU vs Time of curcumin at 2 different concentrations against *C.jejuni* 81176. Curcumin was not effective at reducing or killing the bacteria within 24 hours.

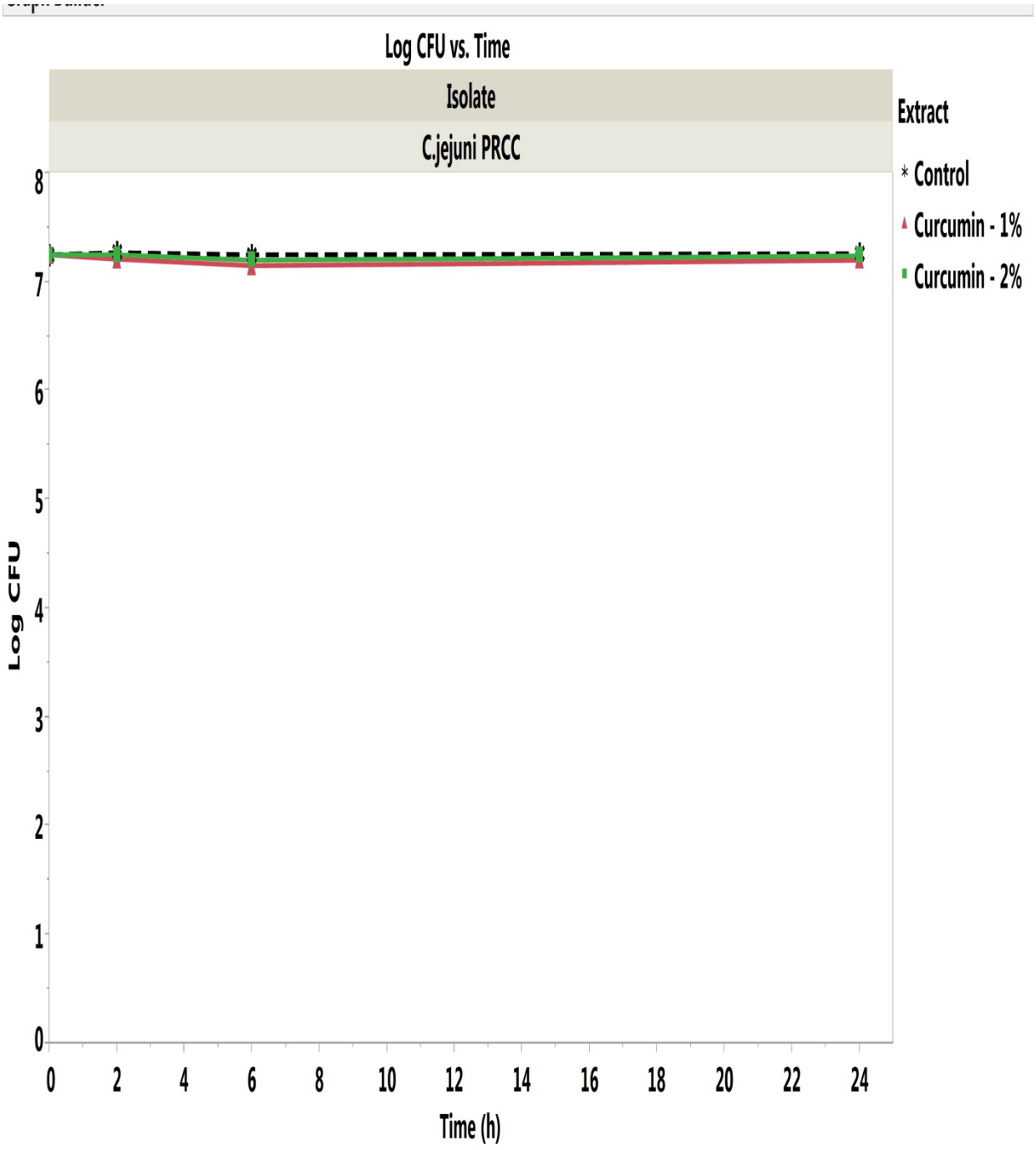

Figure A1.2. Log CFU vs Time of curcumin at 2 different concentrations against *C.jejuni* PRCC. Curcumin was not effective at reducing or killing the bacteria within 24 hours.

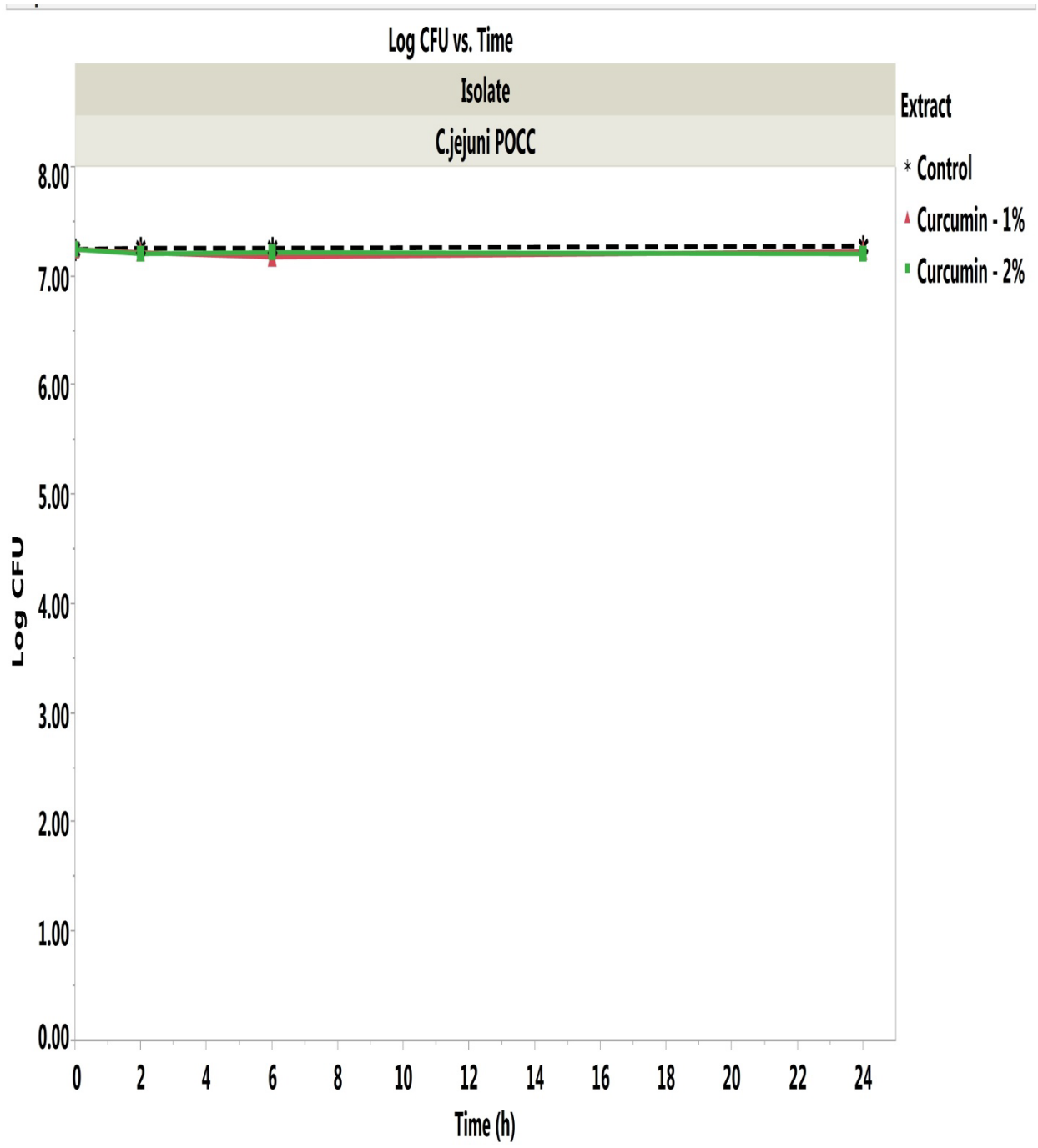

Figure A1.3. Log CFU vs Time of curcumin at 2 different concentrations against *C.jejuni* POCC. Curcumin was not effective at reducing or killing the bacteria within 24 hours.

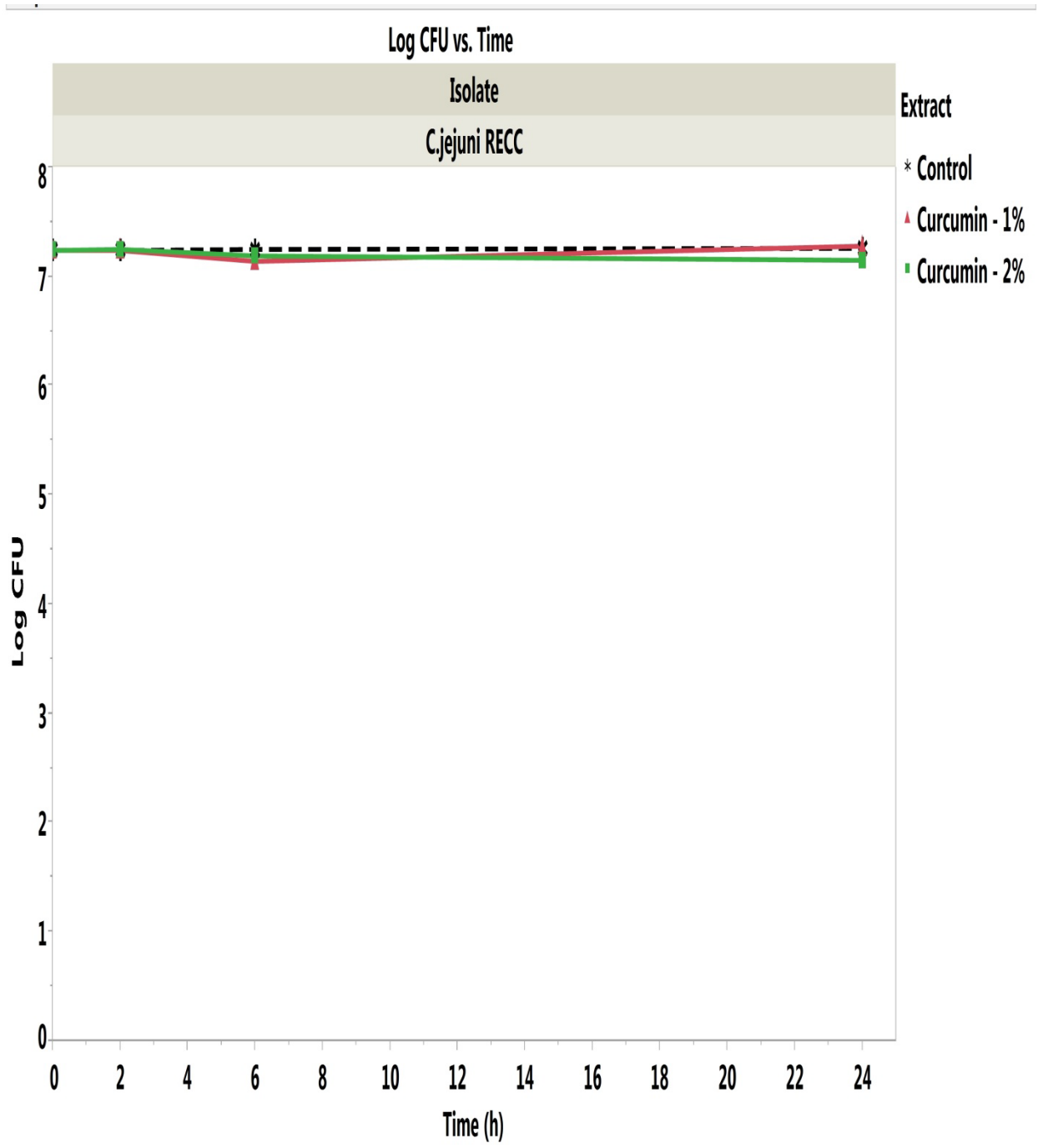

Figure A1.4. Log CFU vs Time of curcumin at 2 different concentrations against *C.jejuni* RECC. Curcumin was not effective at reducing or killing the bacteria within 24 hours.

APPENDIX A2

COMMERCIALLY AVAILABLE EXTARCTS PRODUCTION PROCESS

INTRODUCTION

The plant extracts are extracted from the different parts of the plants using various methods and are purified to meet high standards required of commercial products.

METHODS

Right + Right Method* [2]

If constituents are water soluble the extracts are extracted by dissolving in water otherwise hydroethanolic extraction processes are used. If fat soluble constituents are required to be captured a supercritical extraction technique is used.

Low + Low method* [1]

Once the bioactive constituents of the herbs have been extracted, the extracts are then highly concentrated with low + low method—low temperature in combination with low atmospheric pressure—to protect the fragile, subtle vital forces of each plant.

Preserving the full chemical makeup requires a slow, careful concentration technique involving low temperature and low atmospheric pressure.

The low + low method of concentration uses a low atmospheric pressure of 24 millibars (versus the Earth's standard sea level pressure of 1,013.25 millibars) that allows the water and alcohol to evaporate slowly (much more slowly than other conventional concentration methods), while a low temperature of 60°C (140°F, nearly 75° below water's boiling point of 212°F/100°C) prevents any volatile, subtle, natural compounds from being damaged. As it evaporates, the

*Method described in this page are from gaia® HERBS, 101 Gaia Herbs, Brevard, NC 28712

organic grain alcohol is recaptured and purified, creating a closed-loop system in which no alcohol evaporates into the environment.

EXTRACTS

Oregano Leaf Extract

- 333 mg/ml herb equivalency
- Extraction method is Right+ Right

Green Tea Leaf Extract

- 500 mg/ml herb equivalency
- Extraction method is Right+ Right
- Low + Low Concentration method

Hawthorn berry, flower and leaf

- 667 mg/ml herb equivalency
- Extraction method is Right+ Right
- Low + Low Concentration method

Curcumin

- 95% total curcuminoid content from turmeric rhizome

*Method described in this page are from gaia® HERBS, 101 Gaia Herbs, Brevard, NC 28712

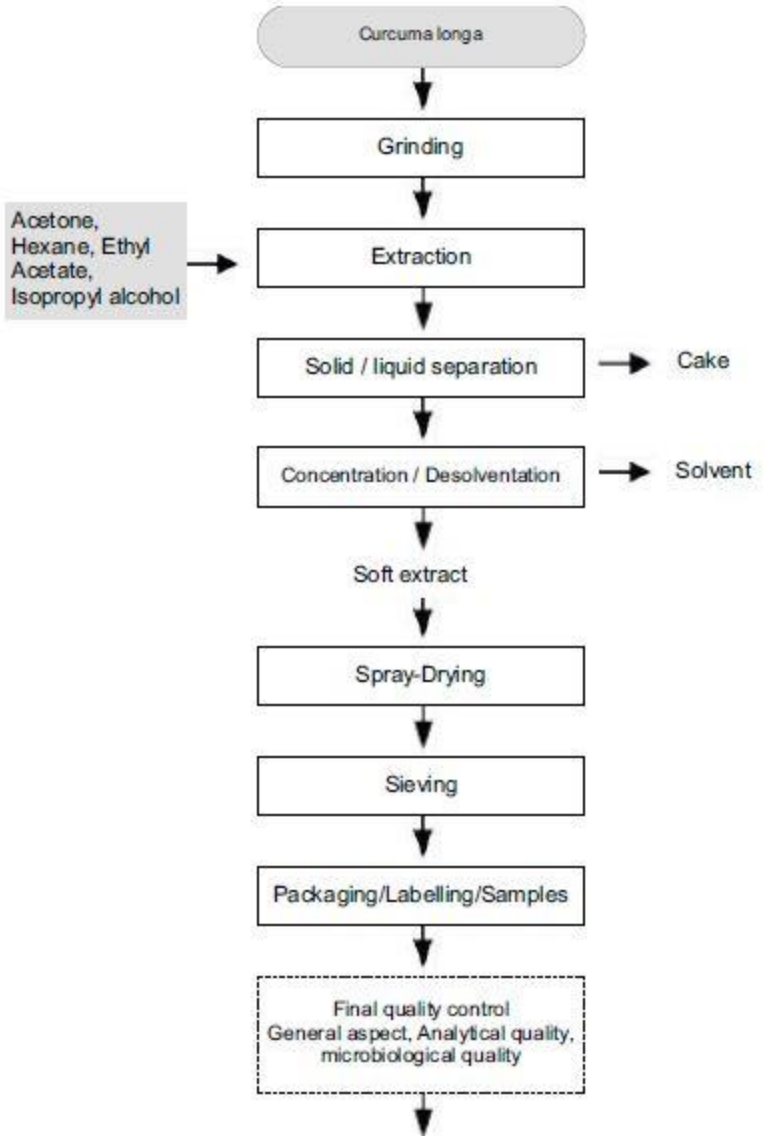

Figure A2.1 Curcumin commercial extraction process** (Source: G.Harris, personal communication, April 16, 2014)

**Method described in this flow chart is courtesy of Alfa Aesar, A Johnson Matthey company

REFERNCES

1. Low + Low Method. Retrieved August 31, 2014, from http://www.gaiaherbs.com/pages/process_step/6/Low-Low-Method
2. Right + Right Method. Retrieved August 31, 2014, from http://www.gaiaherbs.com//pages/process_step/5/RightRight-Method