Effects of Chili Pepper Extracts on Microbial Viability and Growth

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Morrine Auma Omolo

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Dedication

- ✓ To my husband, for believing in my dreams and helping me achieve them
- ✓ To my mom, for being an unparalleled role-model of hard work and dedication
- ✓ To my siblings, for enriching my life in ways words cannot express
- ✓ To my late father and brother, both of whom I miss dearly

Abstract

Although there are hundreds of varieties of chili peppers, those of the *Capsicum annuum* are more readily available. However, the more promising varieties such as *Capsicum Chinense* have not been extensively investigated. This study, the capsaicin content of 29 chili peppers grown under the same conditions is determined. 24 of the samples belong to the *Capsicum chinense* species while the others belong to the *C. annuum* species. Several samples from similar pepper plants of the *C. chinense* species showed wide variation in capsaicin content. The methanol extracts of the 29 samples were then tested for antimicrobial effects against well-known foodborne pathogens and one commensal fungus. The resazurin assay tested for bactericidal properties while the growth inhibition assay tested for bacteriostatic properties. The samples high in capsaicin showed antimicrobial properties, while no effects of bacterial viability and growth was noted from the samples low in capsaicin, except for the Tobago Scotch Bonnet Red pepper.

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1 General Introduction

The FDA Food Safety Modernization Act (FSMA), the most sweeping reform of our food safety laws in more than 70 years, was signed into law by President Obama on January 4, 2011. It aims to ensure the U.S. food supply is safe by shifting the focus from responding to contamination to preventing it. The Preventive Controls for Human Food rule and Preventive Controls for Animal Food rule are now final, and compliance dates for some businesses begin in September 2016. The preventive controls final rules announced on September 10 2015, are the result of an extensive outreach effort, and incorporate thousands of public comments, including valuable input from farmers, consumers, the food industry and academic experts, to create a flexible and targeted approach to ensure food safety (1).

The production of high-quality, safe (pathogen-free) food relies increasingly on natural sources of antimicrobials to inhibit food-spoilage organisms, foodborne pathogens and toxins. The demand for biocides in the food industry is growing as many consumers demand products that are preserved naturally. The discovery and development of new antimicrobials from natural sources for a wide range of applications requires that knowledge of traditional sources for food antimicrobials is combined with the latest technologies in identification, characterization and application (2).

Past research suggests that some chili peppers contain capsaicin, an antimicrobial compound. However, these studies have been carried out in small scale, with an average of four varieties of chili peppers. Consequently, the results are promising, but a more extensive study on the antimicrobial properties of chili peppers is necessary to corroborate what the Mayans might have inadvertently discovered (3).

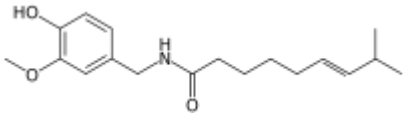
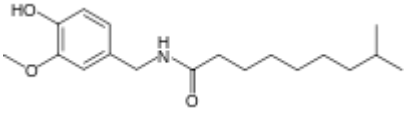
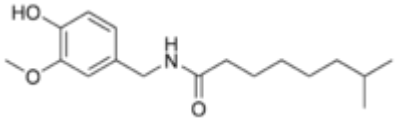
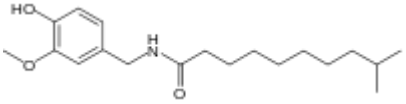
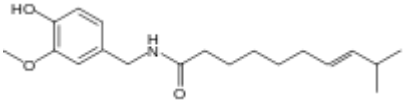
2 Literature Review

2.1 Introduction

Human use of chili peppers dates back to prehistoric times. Preserved peppers have provided evidence that South Americans ate and grew *aji*, (chili in English), in 2500 B.C. The peppers became increasingly common and integrated into the diet of particular cultures. However, chili peppers and similar spices remained isolated in these cultures until the 13th century, when they became available to civilizations throughout the world (4).

The pungency of chili peppers is due to the accumulation of capsaicinoids (also known as capsinoids), a group of naturally produced compounds that are unique to the *Capsicum* genus (Table 1) (5). The chili pepper is a member of the *Solanaceae* family. It is a diploid, facultative, self-pollinating crop, and closely related to potato, tomato, eggplant, tobacco and petunia. It is one of the oldest domesticated crops in the Western hemisphere, the most widely grown spice in the world, and is a major ingredient in most global cuisines. *Capsicum* species are commonly grown in warm humid regions such as the tropics and subtropics and their fruits are mainly used in local cuisines (6, 7).

Table 1: Chemical structure of the six most abundant capsaicinoids in chili peppers

Capsaicinoid name	Chemical structure
Capsaicin	
Dihydrocapsaicin	
Nordihydrocapsaicin	
Homodihydrocapsaicin	
Homocapsaicin	

Chili peppers are widely used as spices in traditional Mexican foods. The flavor and pungent power of these peppers varies widely and so do their contents of capsaicin and its capsaicinoid analogs (6). When eaten, many chili peppers evoke a sensation of heat and/or pain to the neurological systems in mammals, and these adverse effects can be overcome through the consumption of foods containing casein such as milk, cheese, or yogurt. Studies of the botanical pharmacopoeia of the indigenous Mayan inhabitants of Mesoamerica have shown that chili peppers (*Capsicum* species) are incorporated into a number of medicinal preparations. These preparations were applied for a variety of ailments including respiratory problems, bowel complaints, earaches, and sores. Early European observers noted the omnipresent nature of chili peppers in the Mayan diet, reporting that nothing was eaten without them. While typically regarded as a spice, the substantial role that chili peppers occupy in this culture's diet may have important nutritional consequences for these people (4).

Chili peppers have a wide range of uses, including pharmaceutical, natural coloring agents and cosmetics, as an ornamental plant, and as the active ingredient in most defense repellants (i.e. pepper sprays). Capsaicin, a well-studied chemical component of the *Capsicum* species and one of the pungent capsaicinoids found in chili peppers, has already demonstrated a high degree of biological activity affecting the nervous, cardiovascular, and digestive systems. Chemical analysis has demonstrated that *Capsicum* fruits contain relatively high concentrations of several essential nutrients, including vitamin C (up to 6 times the concentration of an orange) (8).

Strong consumer demand for safe and high-quality foods can be attributed in part to the wide spread availability and accessibility of quality health data and information. There are also new concerns about food safety due to increasing occurrences of new food-borne disease outbreaks caused by pathogenic microorganisms. This raises considerable challenges, particularly since there is increasing unease regarding the use of chemical preservatives and artificial antimicrobials to inactivate or inhibit growth of spoilage and pathogenic microorganisms (9). In addition, currently available treatment options for foodborne pathogen infections have drug - related side effects, bacterial resistance to antimicrobials, and in some cases no medical treatment exists for organisms such as *Escherichia coli* O157:H7.

Therefore, newer treatments which are safe, cost effective, and simple to administer are urgently needed. In light of this, the use of nutritional agents is an attractive alternative to conventional therapeutics and warrants further investigation (6). Consequently, natural antimicrobials such as chili peppers are receiving a good deal of attention for a number of microorganism-control issues (9). Recent reports state that the *Capsicum* genus, among other plant genera, is a good source of antimicrobial and antifungal compounds (10).

2.2 Species of the Genus *Capsicum* presently known

Capsicum species are small perennial herbs native to tropical South America. The majority of researchers believe that this genus is comprised of more than 20 species. The 5 most common ones believed to be a result of domestication are *C. annuum, C.*

baccatum, *C. frutescens*, *C. chinense* and *C. pubescens* (8), (Figure 1). The other species are exotic and not as widely distributed as these five.

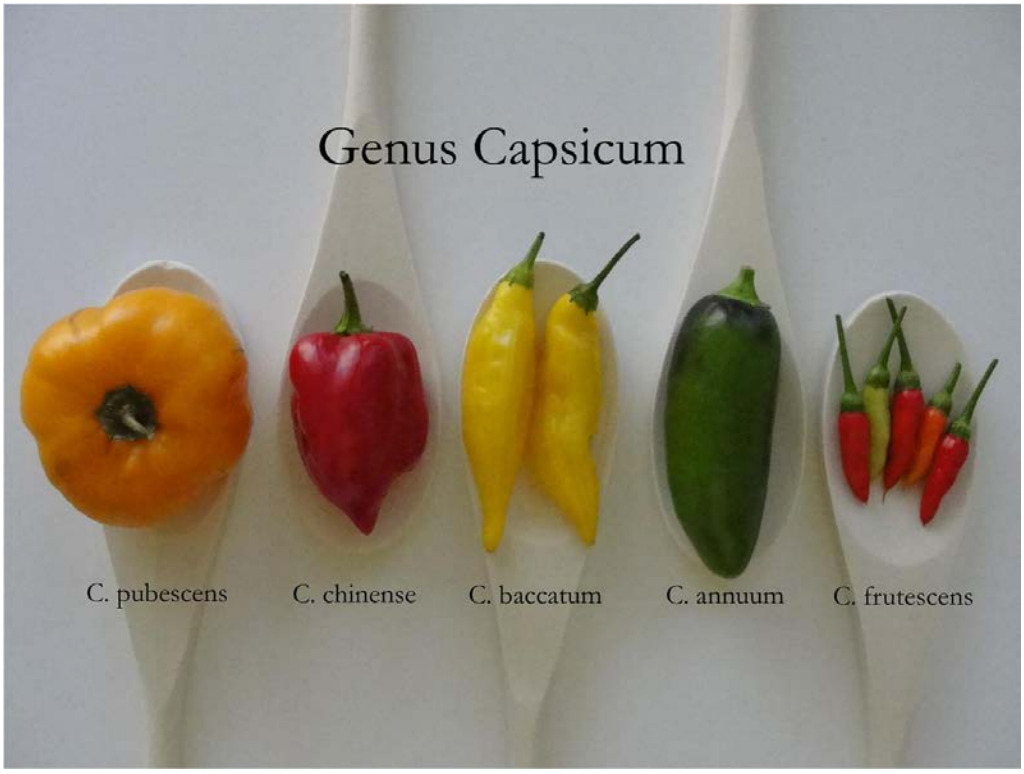


Figure 1: Most common members of Genus *Capsicum*

Courtesy of Dr. D. J. Baumler

Below is a list of the other presently known species (11).

- *Capsicum buforum*
- *Capsicum campylopodium*
- *Capsicum cardenasii*
- *Capsicum ceratocalyx*
- *Capsicum chacoense*
- *Capsicum coccineum*
- *Capsicum cornutum*
- *Capsicum dimorphum*
- *Capsicum dusenii*
- *Capsicum eximium*

- *Capsicum flexuosum*
- *Capsicum friburgense*
- *Capsicum galapagoense*
- *Capsicum geminifolium*
- *Capsicum havanense*
- *Capsicum hookerianum*
- *Capsicum hunzikerianum*
- *Capsicum lanceolatum*
- *Capsicum leptopodum*
- *Capsicum lycianthoides*
- *Capsicum minutiflorum*
- *Capsicum mirabile*
- *Capsicum mositicum*
- *Capsicum parvifolium*
- *Capsicum pereirae*
- *Capsicum ramosissimum*
- *Capsicum recurvatum*
- *Capsicum rhomboideum*
- *Capsicum schottianum*
- *Capsicum scolnikianum*
- *Capsicum spina-alba*
- *Capsicum stramoniifolium*
- *Capsicum tovarii*
- *Capsicum villosum*

2.3 Top 14 Foodborne pathogens

According to the U.S Food and Drug and Administration (FDA), there are several foodborne pathogens that are of concern and harmful to the general public, and are particularly harmful to pregnant women (Table 2) (12).

Aside from these 14, there are other well-known pathogens some of which are foodborne, including *Bacillus cereus*, *Enterobacter aerogenes* (8), *Pseudomonas aeruginosa* (8, 13) and *Helicobacter pylori* (14) which seem to be of interest to research scientists.

2.4 Studies on antimicrobial effects of chili pepper extracts on some foodborne and/or human pathogens

2.4.1 *Bacillus subtilis* (not typically associated with foodborne illness)

According to Molina-Torres *et al*. (15), capsaicin (pure, purchased from Sigma Aldrich), had a strong inhibitory effect towards *B. subtilis* starting from 25 µg/ml (minimum concentration assayed).

2.4.2 *Escherichia coli*

Molina-Torres *et al*. (15) determined that capsaicin (pure, purchased from Sigma Aldrich), at concentrations up to 200 or 300 µg/ ml only retarded the growth of *E. coli.*

2.4.3 *Salmonella* Typhimurium

Careaga *et al*. (13) investigated the antimicrobial effect of *Capsicum* extract on *Salmonella* Typhimurium inoculated in minced beef. The minimum lethal concentration of the pepper extract was 1.5 ml/100 g of meat. The combination of sodium chloride and *C. annum* extract tested was not successful to eliminate *Salmonella.* This could be explained by the fact that *Salmonella* is tolerant to salt. The researchers proposed using a combination that had less salt and more pepper extract, because any more salt would be too much to eat.

2.4.4 *Pseudomonas aeruginosa*

In the same study, Careaga *et al*. (13) investigated the antimicrobial effect of *Capsicum* extract on *P. aeruginosa* inoculated in minced beef. A reduction of *P. aeruginosa* growth was observed between 0.06-0.1 ml/100 g meat, with a bacteriostatic

effect between 0.5-1.5 ml/100 g meat. As the extract concentration increased, a drastic bactericidal effect was observed, particularly between 4-5 ml/100 g meat. The combination of sodium chloride and *C. annum* extract tested eliminated *P. aeruginosa* after 3 days of storage.

Table 2: Top 14 food borne pathogens, according to the FDA

Pathogen	Basics	Sources	Symptoms	Incubation	Duration
Campylobacter jejuni	A bacterium that's the most common bacterial cause of diarrhea in the U.S. Must-Know: Children under age 1 have the highest rate of Campylobacter infections. Unborn babies and infants are more susceptible on first exposure to this bacterium. In addition, there's a low threshold for seeking medical care for infants.	Raw milk, untreated water, raw and undercooked meat, poultry, or shellfish	Diarrhea (sometimes bloody), stomach cramps, fever, muscle pain, headache, and nausea.	Generally 2 to 5 days after eating contaminated food	7 to 10 days
Clostridium botulinum	A bacterium that can be found in moist, low-acid food. It produces a toxin that causes botulism, a disease that causes muscle paralysis. Must-Know: Don't feed a baby honey - at least for the first year. Honey can contain Clostridium botulinum spores. Infant botulism is caused by consuming these spores, which then grow in the intestines and release toxin.	Home-canned and prepared foods, vacuum-packed and tightly wrapped food, meat products, seafood, and herbal cooking oils	Dry mouth, double vision followed by nausea, vomiting, and diarrhea. Later, constipation, weakness, muscle paralysis, and breathing problems may develop. Botulism can be fatal. It's important to seek immediate medical help.	4 to 36 hours after eating contaminated food	Recovery can take between 1 week to a full year.
Clostridium perfringens	A bacterium that produces heat-stable spores, which can grow in foods that are undercooked or left out at room temperature.	Meat and meat products	Abdominal pain, diarrhea, and sometimes nausea and vomiting.	8 to 12 hours after eating contaminated food	Usually 1 day or less

Pathogenic *Escherichia coli (E. coli)*	A group of bacteria that can produce a variety of deadly toxins.	Meat (undercooked or raw hamburger), uncooked produce, raw milk, unpasteurized juice, and contaminated water	Severe stomach cramps, bloody diarrhea, and nausea. It can also manifest as non-bloody diarrhea or be symptomless. Must-Know: It can cause permanent kidney damage which can lead to death in young children.	Usually 3 to 4 days after ingestion, but may occur from 1 to 10 days after eating contaminated food.	5 to 8 days
Listeria monocytogenes	A bacterium that can grow slowly at refrigerator temperatures. Must-Know: Listeria can cause serious illness or death in pregnant women, fetuses, and newborns.	Refrigerated, ready-to-eat foods (meat, poultry, seafood, and dairy - unpasteurized milk and milk products or foods made with unpasteurized	Fever, headache, fatigue, Muscle aches, nausea, vomiting, diarrhea, meningitis, and miscarriages.	48 to 72 hours after ingestion, but may occur from 7 to 30 days after eating contaminated food.	1 to 4 days.
Norovirus (Norwalk-like Virus)	A virus that's becoming a health threat. It may account for a large percent of non- bacterial foodborne illnesses.	Raw oysters, shellfish, coleslaw, salads, baked goods, frosting, contaminated water, and ice. It can also spread via person-to- person.	Diarrhea, nausea, vomiting, stomach cramps, headache, and fever.	24 to 48 hours after ingestion, but can appear as early as 12 hours after exposure.	1 to 2 days
Salmonella enteritidis	A bacterium that can infect the ovaries of healthy-appearing hens and internally infect eggs before the eggs are laid.	Raw and undercooked eggs, raw meat, poultry, seafood, raw milk, dairy products, and produce	Diarrhea, fever, vomiting, headache, nausea, and stomach cramps Must-Know: Symptoms can be more severe in people in at- risk	12 to 72 hours after eating contaminated food	4 to 7 days

Salmonella Typhimurium	Some strains of this bacterium, such as DT104, are resistant to several antibiotics.	Raw meat, poultry, seafood, raw milk, dairy products, and produce	Diarrhea, fever, vomiting, headache, nausea, and stomach cramps Must-Know: Symptoms can be more severe in people in the at-risk groups, such as pregnant women.	12 to 72 hours after eating contaminated food	4 to 7 days
Shigella	A bacterium that's easily passed from person-to-person via food, as a result of poor hygiene, especially poor hand washing. Only humans carry this bacterium.	Salads, milk and dairy products, raw oysters, ground beef, poultry, and unclean water	Diarrhea, fever, stomach cramps, vomiting, and bloody stools	1 to 7 days after eating contaminated food	5 to 7 days
Staphylococcus aureus	This bacterium is carried on the skin and in the nasal passages of humans. It's transferred to food by a person, as a result of poor hygiene, especially poor hand washing. When it grows in food, it makes a toxin that causes illness.	Dairy products, salads, cream-filled pastries and other desserts, high-protein foods (cooked ham, raw meat and poultry), and humans (skin, infected cuts, pimples, noses, and throats)	Nausea, stomach cramps, vomiting, and diarrhea	Usually rapid - within 30 minutes to 8 hours after eating contaminated food	24 to 48 hours
Vibrio cholerae	A bacterium that occurs naturally in estuarine environments (where fresh water from rivers mix with salt water from oceans). It causes cholera, a disease that can cause death if untreated.	Raw and undercooked seafood or other contaminated food and water.	Often absent or mild. Some people develop severe diarrhea, vomiting, and leg cramps. Loss of body fluids can lead to dehydration and shock. Without treatment, death can occur within hours.	6 hours to 5 days after eating contaminated food	7 days

Vibrio parahemolyticus	A bacterium that lives in saltwater and causes gastrointestinal illness in people.	Raw or undercooked fish and shellfish	Diarrhea, stomach cramps, nausea, vomiting, headache, fever, and chills	4 to 96 hours after eating contaminated food	2.5 days
Vibrio vulnificus	A bacterium that lives in warm seawater. It can cause infection in people who eat contaminated seafood or have an open wound exposed to seawater.	Raw fish and shellfish, especially raw oysters	Diarrhea, stomach pain, nausea, vomiting, fever, and sudden chills. Some victims develop sores on their legs that resemble blisters.	Usually within 16 hours after eating contaminated food or exposure to organism	2 to 3 days
Yersinia enterocolitica	A bacterium that causes yersiniosis, a disease characterized by diarrhea and/ or vomiting.	Raw meat and seafood, dairy products, produce, and untreated water	Fever, diarrhea, vomiting, and stomach pain Must-Know: Symptoms may be severe for children.	1 to 2 days after eating contaminated food	1 to 2 days

Adopted from the FDA website (12)

Table 3: *In vitro* ciprofloxacin/capsaicin studies, against *Staphylococcus aureus* strains

		MIC (mg/ml of ciprofloxacin for respective strain with/without test molecule (fold reduction)		
Capsaicin (mg/L)	MIC of capsaicin	SA-1199	SA-1199B	SA-1758
Capsaicin (50)	>100	0.12/0.25 (2)	2/8 (4)	0.125/0.125 (0)
Capsaicin (25)	>100	0.12/0.25 (2)	2/8 (4)	0.125/0.125 (0)
Capsaicin (12.5)	>100	0.25/0.25 (0)	4/8 (2)	0.125/0.125 (0)
Reserpine (25)	>100	0.12/0.25 (2)	2/8 (4)	0.125/0.125 (0)

Table adopted from Kalia *et al*. (16)

Table 4: PAE of ciprofloxacin alone and in combination with capsaicin against *Staphylococcus aureus* SA-1199B after exposure of 2 h

	Mean PAE[1] (h) ± S.D		
Regimen	0.25×MIC (2	0.5×MIC (4 mg/L)	MIC (8 mg/L)
Ciprofloxacin	0.3 ± 0.1	1.0 ± 0.1	1.3 ± 0.17
Ciprofloxacin + Capsaicin (25mg/L)	1.0 ± 0.2	1.5 ± 0.17	2.4 ± 0.2

[1]Post Antibiotic Effect

Table adopted from Kalia *et al*. (16)

Table 5: Mutation frequency of *Staphylococcus aureus* ATCC 29213

Capsaicin (mg/L)	**2×MIC[1] (0.5 mg/L)**	**4×MIC[1] (1 mg/L)**	**8×MIC[1] (2 mg/L)**	**16×MIC[1] (4 mg/L)**
0	1.47×10-9	7.7×10-9	4.3×10-9	<10-9
12.5	13.5×10-9	3.9×10-9	<10-9	<10-9
25	2.5×10-9	< 10-9	<10-9	<10-9

[1]Minimum Inhibitory Concentration

Table adopted from Kalia *et al*. (16)

2.4.5 *Staphylococcus aureus*

Nitin *et al*. (16), evaluated the possibility of capsaicin acting as an inhibitor of the NorA efflux pump of *Staphylococcus aureus*. The minimum inhibitory concentration (MIC) of ciprofloxacin was reduced 2 to 4 fold in the presence of capsaicin. This reduction was more prominent for *Staphylococcus aureus* SA-1199B (NorA overproducing) as compared with *Staphylococcus aureus* SA-1199 (wild-type) up to 25 mg/L capsaicin. Beyond that, no concentration dependent effect was observed. *Staphylococcus aureus* SA-K1758 (*norA* knockout) showed no reduction in the MIC of ciprofloxacin. Table 3 shows *in vitro* ciprofloxacin/ capsaicin combination studies. Table 4 shows post- antibiotic effect (PAE) of ciprofloxacin alone and in combination with capsaicin against *Staphylococcus aureus* SA-1199B after exposure of 2 h. Ciprofloxacin at 4 mg/L, at which no mutant was selected, was defined as the mutant prevention concentration (MPC). When tested in combination with capsaicin at 12.5 and 25 mg/L, the MPC of ciprofloxacin was reduced to 2 and 1 mg/L, respectively. The MPC of the combination was found to be lower than the C_{max} of the ciprofloxacin (3-4 mg/L), indicating the clinical relevance of these combinations in restricting the selection of resistant mutants. Ethidium bromide fluoresces only when it is bound to nucleic acids inside cells. Only the control cells without capsaicin extruded ethidium bromide, resulting in a significant decrease in florescence over the assay period. In the presence of capsaicin, the loss of florescence was significantly reduced, reflecting a strong interference with ethidium bromide efflux by capsaicin. Table 5 shows the mutation frequency of *Staphylococcus aureus* ATCC 29213 (16).

2.4.6 *Vibrio cholerae*

This study examines common spices to determine their inhibitory capacity against virulence expression of *V. cholera* (Table 6). Among them methanol extracts of red chili, sweet fennel and white pepper could substantially inhibit cholera toxin (CT) production (Table 7). As these species act against virulence expression rather than viability of *V. cholerae,* there is a lesser chance of developing resistance (17).

In a different study, Chatterjee *et al*. (18), determined that the methanol extract of red chili, and purified capsaicin could inhibit cholera toxin (CT) production in recently emerged *V. cholerae* O1 El Tor variant strains without affecting their viability. All 23 strains of *V. cholerae* used in the study, (Table 8), were grown in the lab. Crude methanol extract of the red chili pepper was used (individual ingredients not isolated). Capsaicin was purchased from LKT laboratories Inc., MN. RNA isolation and real-time transcription-PCR (qRT-PCR) assay revealed that capsaicin effectively repressed the transcription of *ctxA*, *tcpA*, and *toxA* genes, but not the *toxR* and *toxS* genes. It enhanced the transcription of the gene *hns* (Table 9).

Based on the experimental results, the researchers proposed a mechanism by which capsaicin and the red chili methanol extract represses the virulence genes of *V. cholerae*. Briefly, the activation of *toxR*, *toxS*, *tcpP*, and *tcpH* is caused by environmental factors such as pH, temperature, and osmolarity. This activation subsequently activates *ctxAB* and *tcpA* transcriptions via activation of transcriptional activator *toxT*. HN-S is a basal repressor of *toxT*, *ctxAB* and *tcpA* genes under non-permissive conditions. In the presence of capsaicin, while *ctxAB*, *tcpA*, and *toxT* transcriptions were repressed, the transcription of *hns* was enhanced. Capsaicin may probably repress the virulence genes

transcriptions in a direct manner or via modulation of the global regulator *hns* gene. The higher inhibitory impact of red chili methanol extract than capsaicin (43- and 23- fold respectively) indicates the possibility of other unidentified compound(s) in red chilis that can directly inhibit or synergistically act with capsaicin (18).

2.4.7 *Helicobacter pylori*

In their experiment, Jones *et al*. (7) determined that capsaicin inhibited growth of *H. pylori* strain LC-11 in a dose-dependent manner at concentrations above 10 µg/ml (ANOVA, $P < 0.05$). This bactericidal effect was evident within 4 h of incubation. After 24 h, growth of the bacteria was completely inhibited. The effect of capsaicin was maximal at a concentration of 50 µg/ml. This bactericidal effect was not limited to *H. pylori* LC-11. Growth of LC-32 and LC-28 were inhibited to a similar extent at 500 µg/ml.

To examine the possible influence of pH on the bactericidal activity of capsaicin, the growth of *H. pylori* strain LC-11 was compared in broth culture at pH 4.5, 5.4, and 6.4 in the presence and absence of capsaicin. At each of these pH values, the growth of *H. pylori* was inhibited compared to bacterial growth in standard broth culture at pH 7.38. Capsaicin exerted a growth inhibitory effect of 92 ± 3.7% at pH 5.4 and 72 ± 11% at pH 6.4. At pH 4.5, bacterial growth did not differ in the presence (93.5 ± 2.4%) and absence (88.4 ± 7.8%) of capsaicin (7).

2.4.8 *Listeria monocytogenes*

Reverse-phase HPLC analysis was performed to determine the capsinoid-content of the pepper extracts of habanero, serrano, and pimiento chili peppers. Table 10 shows

the HPLC profile of standard phenylpropanoid compounds, capsaicin, and dihydrocapsaicin from chili extracts, while Table 11 shows the content of some capsinoids in the habanero, serrano, and pimiento moron extracts (mg/ml) (6). Lidia *et al*. do not specify what serotypes of the peppers they used. Figure 2 shows pictures of the most readily available varieties in the market.

The capsinoid compositions of the three pepper extracts are different, and this may influence their antimicrobial effect. The concentration of capsaicin and capsaicinoids used in this study did not show an inhibitory effect on *L. monocytogenes*. Habanero which has the highest content of capsaicin was the least effective as a bacterial inhibitor. The pimiento morron extract, which contains m-coumeric acid and cinnamic acid but no capsaicin, showed a good inhibitory effect on the bacterium (Table 12). (6)

Table 6: Natural compounds identified to act against diarrheagenic *Vibrio* spp.

Plant	**Scientific name**	**Specific compound**	**Target**	**Mechanism**
Wasabi	Wasabi japonica	Allyl isothiocynate	*V. parahemolyticus*	Inhibit growth
Green tea	Camellia sinensis	Catechins	*V. cholera*	Inhibit growth and CT[1]
Guazuma	Guazuma ulimifolia	Procyanidins	*V. cholera*	CT activity
Daio (Kampo formulation)	Rhei rhizome	Gallate analogues	*V. cholera*	CT activity
Apple	Malus spp.	Aplephenon	*V. cholera*	CT activity
Hop	Humulus lupulus	Procyanides	*V. cholera*	CT activity
Neem	Azadirachta indica	Unknown	*V. cholera*	Inhibit growth
Elephant garlic	Allium	Oil (diallyl sulfides)	*V. cholera*	Inhibit growth
Red bayberry	Myrica rubra	Unknown	*V. cholera*	Inhibit CT production
Red chili	*Capsicum annum*	Capsaicin	*V. cholera*	Inhibit CT production

[1]Cytotoxin

Table adopted from Yamasaki *et al*. (17)

Table 7: % Inhibition of CT production in *V. cholerae* O1 E1 Tor variant strains (isolated from cholera patients in India) with methanol extracts of 6 different commonly used spices (100 μg/ml)

Stain ID	**Isolation**	**Red chili**	**Sweet fennel**	**White**	**Red pepper**	**Cassia bark**	**Star anise**
CO 533	1994	97	95	86	68	45	50
CRC 27	2000	97	92	99	80	79	66
CRC 41	2000	90	96	94	53	86	6.0
CRC 87	2000	94	85	87	56	78	29

Table adopted from Yamasaki *et al*. (17)

Table 8: *Vibrio cholerae* strains used in the study

Serial no.	Strain	Serogroup/biotype	ctxB genotype	Country	Isolation
1	NICED-1	O1 El Tor	El Tor	India	1970
2	NICED-10			India	1970
3	NICED-3			India	1980
4	P130			Peru	1991
5	VC190			India	1993
6	VC301	O1 El Tor	Classical	India	1992
7	Al-091	variant		Bangladesh	1993
8	CO533			India	1994
9	CRC27			India	2000
10	CRC41			India	2000
11	CRC87			India	2000
12	B33			Mozambique	2004
13	1’/05			India	2005
14	2’/05			India	2005
15	5’/05			India	2005
16	2680713			Bangladesh	2006
17	2684269			Bangladesh	2006
18	SG24	O139	El Tor	India	1992
19	CRC142		Classical	India	2000
20	VC82	Non-O1/	El Tor	India	1989
21	VC259	Non-O139		India	1991
22	569B	O1 classical	Classical	India	1948
23	O395			India	1964

Table adopted from Chatterjee *et al*. (18)

Table 9: Primers and probes used for qRT-PCR

Primer/probe	Primer and probe sequence (5' – 3')	Amplicon size
ctxA F	GGA GGG AAG AGC CGT GGA T	
ctxA P	CAT CAT GCA CCG CCG GGT TG	66
ctxA R	CAT CGA TGA TCT TGG AGC ATT C	
tcpA F	GGG ATA TGT TTC CAT TTA TCA ACG T	
tcpA P	TGC TTT CGC TGC TGT CGC TGA TCT T	82
tcpA R	GCG ACA CTC GTT TCG AAA TCA	
toxT F	TGA TGA TCT TGA TGC TAT GGA GAA A	
toxT P	TAC GCG TAA TTG GCG TTG GGC AG	107
toxT R	TCA TCC GAT TCG TTC TTA ATT CAC	
toxR F	GCT TTC GCG AGC CAT CTC T	
toxR P	CTT CAA CCG TTT CCA CTC GGG CG	65
toxR R	CGA AAC GCG GTT ACC AAT TG	
toxS F	TGC CAT TAG GCA GAT ATT TCA CA	
toxS P	TGA CGT CTA CCC GAC TGA GTG GCC C	72
toxS R	GCA ACC GCC CGG CTA T	
tcpP F	TGG TAC ACC AAG CAT AAT ACA GAC TAA G	
tcpP P	TAC TCT GTG AAT ATC ATC CTG CCC CCT GTC	100
tcpP R	AGG CCA AAG TGC TTT AAT TAT TTG A	
tcpH F	GCC GTG ATT ACA ATG TGT TGA GTA T	
tcpH P	TCA ACT CGG CAA AGG TTG TTT TCT CGC	82
tcpH R	TCA GCC GTT AGC AGC TTG TAA G	
hns F	TCG ACC TCG AAG CGC TTA TT	
hns P	CTG CGC TAT CAG GCG AAA CTA AAA CGA AA	70
hns R	GGT GCA CGT TTG CCT TTT G	
recA F	CAA TTT GGT AAA GGC TCC ATC AT	
recA P	CTT AGG CGA CAA CCG CGC	71
recA R	CCG GTC GAA ATG GTT TCT ACA	

Table adopted from Chatterjee *et al*. (18)

Table 10: HPLC profile of standard phenylpropanoid compounds, capsaicin, and dihydrocapsaicin from chili extracts

Compound	**Retention Time (min)***	**% Acetonitrile at which the separation was achieved**
L-phenylalanine	6.55 ± 0.66	27.91
Caffeic Acid	7.00 ± 0.76	28.83
p-coumaric acid + ferulic acid	8.56 ± 0.52	32.03
m-coumaric acid	9.32 ± 0.52	33.59
o-coumaric acid	11.21 ± 0.70	37.46
Trans-cinnamic acid	18.99 ± 094	53.41
Capsaicin	25.72 ± 0.90	67.20
Dihydrocapsaicin	27.33 ± 0.74	70.50

*Data represent an average of ten replicates (± S.D.)

Table adopted from Dorantes *et al*. (6)

Table 11: Content of some capsinoids in the habanero, serrano, and pimiento morron extracts (mg/ml)

Capsinoid	**Habanero**	**Serrano**	**Morron**
o-coumaric acid	0.089 ± 0.01	0.90 ± 0.01	0.18 ± 0.01
m-coumaric acid	-	0.31 ± 0.01	0.21 ± 0.01
Trans-cinnamic acid	-	0.47 ± 0.01	0.21 ± 0.01
Capsaicin	5.88 ± 0.03	0.63 ± 0.01	-
Dihydrocapsaicin	0.86 ± 0.01	0.059 ± 0.01	-

Data represent an average of three replicates (± S.D.)

Table adapted from Lidia *et al*.(13)

Table 12: Zone of growth produced by some phenylpropanoids identified in serrano chili peppers (mm)

Bacteria	***o*-coumaric**	***m*-coumaric**	**Cinnamic acid**	**Capsaicin**	**Dihydro-capsaicin**
B. cereus	Neg	10.0 ± 0.0	8.0 ± 0.8	Neg	Neg
Staphylococcus	Neg	10.0 ± 0.8	6.0 ± 0.8	Neg	Neg
L. monocytogenes	Neg	6.0 ± 0.6	5.0 ± 0.8	Neg	Neg
Salmonella	Neg	2.0 ± 0.8	2.0 ± 0.0	Neg	Neg

Data represents an average of three replicates (± S.D.)

Table adopted from Lidia *et al*. (13)

Figure 2: Habanero, serrano and pimiento peppers

Courtesy of Dr. D. J. Baumler

2.5 Conclusion

As more food scientists, consumers, and members of the medical field gain interest in chili peppers, it is certain that through ethnobotanical observations, *Capsicum* species harbor many economically significant benefits awaiting 'discovery' (4). There are a variety of methods for testing the antimicrobial activities of chili peppers. These methods strongly affect the observed levels of inhibition. Various reasons may contribute in the differences between results, including inconsistency between analyzed plant materials (10).

In these experiments, crude extracts of chili peppers were used; no separation of pepper components was done, except by Dorantes *et al*. (6). Based on the data, it seems that capsaicin had a lesser antimicrobial effect compared to other components of chili pepper extracts. Therefore, future studies should try to determine what compounds in the chili pepper gives the spice its antimicrobial properties, and to do so purification of the

extracts is necessary. Capsaicin gives chili peppers the 'hot' sensation, which some people might not like. It would, therefore, be beneficial if there is another substance in the pepper that could be used in the food industry as a preservative without the pungent taste and hotness.

The studies examined herein were done *in vitro*. However, more tests need to be conducted to determine the antimicrobial effects of chili peppers *in vivo*, especially because such a large number of people eat peppers. This could be a potential means through which to minimize the effect of foodborne pathogens when there is an outbreak. Graham *et al*. (14) were unable to confirm the hypothesis that capsaicin has an inhibitory effect on *H. pylori in vivo*. They believe that natural substances and folk remedies should undergo testing *in vivo* before publication of the *in vitro* results to reduce the possibility of misinforming the public regarding the potential usefulness of these agents.

Varied as these studies may be, they open the doors to greater research on chili peppers. The data already collected and methods of testing offer new directions for future experiments. To obtain more conclusive data, the number of pepper varieties used should be increased since hundreds of thousands of different types of chili pepper plants exist worldwide. The following picture shows some of the most common varieties, including many exotic types sourced from all over the world (Figure 3).

Figure 3: Some common varieties of chili peppers in the market

Courtesy of Dr. D. J. Baumler

For example the six hottest chili peppers in the world, Bhut Jolokia, Trinidad 7-pot, Trinidad Scorpion ButchT, Trinidad Douglah, Trinidad Moruga Scorpion (Figure 4), and Carolina Reaper (not shown), have not been tested and may possess undiscovered antimicrobial compounds and activity (18)

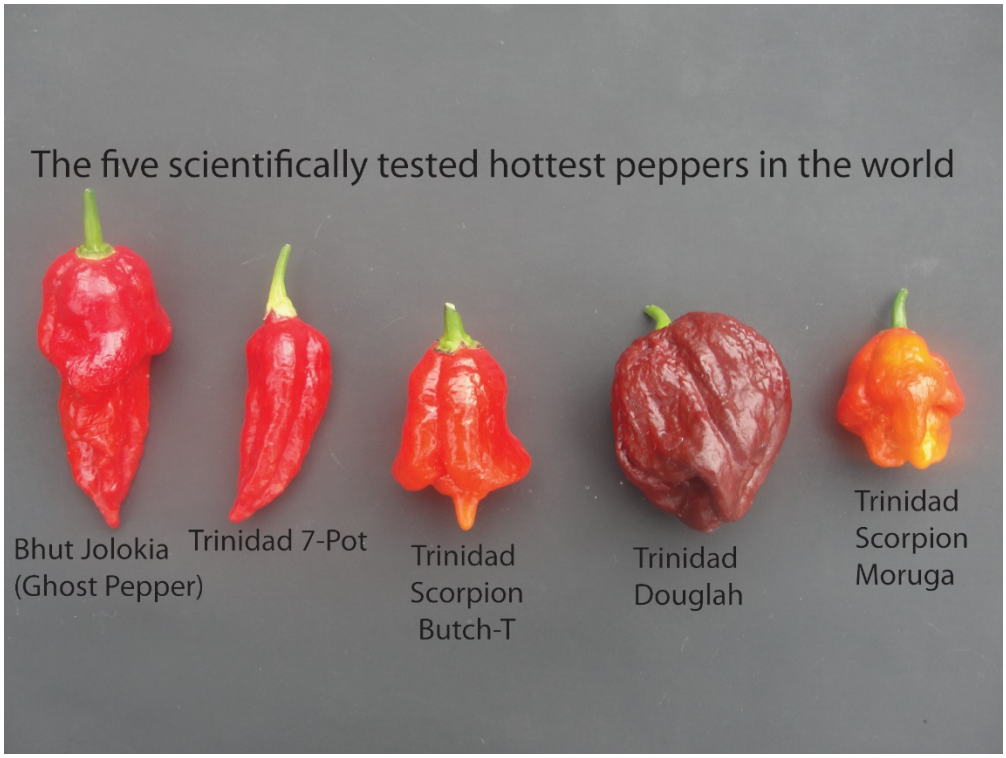


Figure 4: The 5 scientifically tested hottest peppers in the world

Courtesy of Dr. D. J. Baumler

Our lab is working with 29 varieties of chili peppers to determine the antimicrobial effects the extracts of the fruits have on selected foodborne pathogens. These varieties will include peppers with and without capsaicin from all over the world.

3 Determination of the capsaicin content of chili peppers grown under same conditions and subsequent microbial viability studies using the resazurin assay

3.1 Summary

Many plants including chili peppers have been found to possess antimicrobial properties. However, only a few chili pepper varieties have been examined for bactericidal activity and the hottest types in the world, containing the most capsaicin, have never been tested. Resazurin (Alamar Blue), is a tetrazolium-based dye used as an indicator in microbial viability studies. In this study the average capsaicin content of chili peppers grown under the same conditions was determined using a Capsaicin Plate Kit (Cat. # 20-0027). A resazurin assay was performed to investigate bactericidal effects of the chili pepper extracts. Comparisons were then made between the capsaicin and resazurin data to determine whether or not there is a correlation between capsaicin content and effects on microbial viability. 24 of the tested samples are known to contain high levels of capsaicin, while five samples contain little to no capsaicin. Extraction of capsaicin was done using methanol as a solvent. The chili peppers high in capsaicin had bactericidal effects on the microbes tested, in dose dependent manner. The samples low in capsaicin, were not bactericidal.

3.2 Introduction

The emergence of antibiotic resistance has prompted scientist to assiduously research into medicinal plants, not only to ascertain claims of efficacy and safety but also to discover alternative candidates for drug development (19). In this study, chili pepper extracts were examined for bactericidal effects against five microorganisms. The test investigates the effects of the chili pepper extracts on microbial viability, using the resazurin assay method.

Resazurin, also known as Alamar blue, is a dark blue, tetrazolium-based, non-toxic, oxidation-reduction dye which is reduced intracellularly to the pink compound, resorufin, by enzymes in the electron transport system (20-24). The Alamar Blue (AB) assay, which incorporates a redox indicator that turns purple/pink in response to metabolic activity, is commonly used to assess, colorimetrically, the viability and/or proliferation of mammalian cells and micro-organisms. It has been used to non-destructively examine the viability of microorganisms (22, 25-28).

Resazurin salt is dissolved in deionized water, filter-sterilized and added directly to cells in a culture in a homogeneous format. Viable cells with active metabolism reduce resazurin (dark blue) into resorufin (purple/pink). This reaction typically proceeds as shown in Figure 5, when resazurin is exposed to a redox active molecule such as nicotinamide adenine dinucleotide (NADH) (29). Plates are incubated for 2 h: this period should be optimized (kept short enough to avoid reagent toxicity but long enough to provide adequate sensitivity). This method is relatively inexpensive (20, 23).

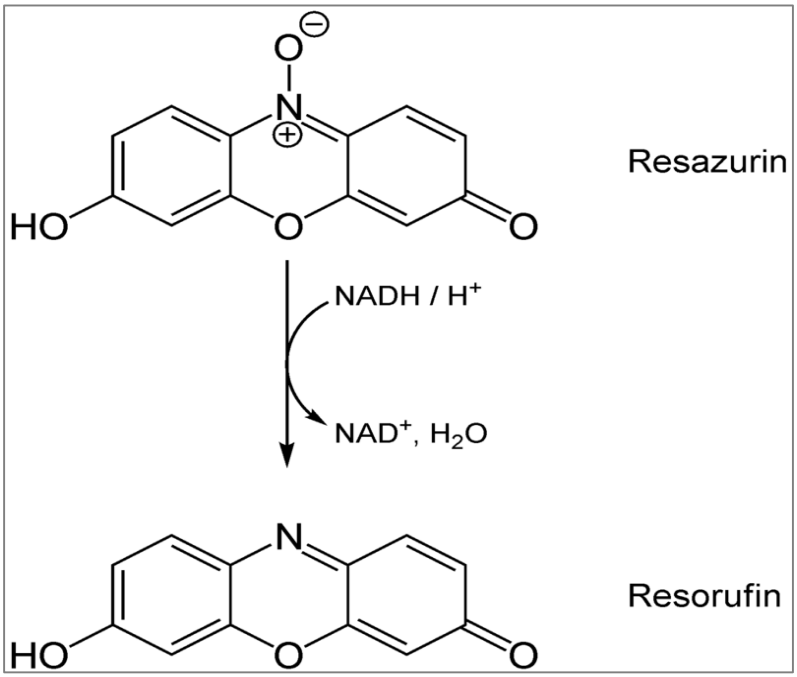


Figure 5: Reduction of resazurin to resorufin (with NADH)

After a 2 h incubation of cells exposed to the chili pepper extracts, the change in color was observed and recorded as N (no change), I (color changed to indigo/purple) or Y (color changed to pink).

3.3 Materials and Methods

3.3.1 Materials

The resazurin dye powder used was obtained from Difco Inc. The Capsaicin Plate Kit (Cat. # 20-0027) was purchased from Beacon Analytical Systems Inc. The capsaicin powder (99.1% pure) was purchased from Chem-impex Int'l Inc. (Wood Dale, IL) and stored at 4^{o}C until use. Stainless steel beads (SSB14B stainless steel beads for

homogenizer bullet blender, 0.9 - 2.0 mm diameter) were purchased from Thomas Scientific. The BBL™ Tryptic Soy Agar (TSA), Becto™ Tryptic Soy Broth (TSB) and BBL™ Brain Heart Infusion (BHI) broth were purchased from Becton, Dickinson and Company (Sparks, MD).

The chili peppers fruits used (Table 13), were cultivated in the University of Minnesota Agricultural fields (St. Paul campus) following the methods of Bosland *et al.* (30), harvested and frozen until samples were prepared.

Table 13: Names, species and seed sources of the chili pepper used in this study

Chili Pepper Cultivar Name	**Species**	**Seed Source**
7-pod Congo SR Gigantic	*C. chinense*	Pepperlover.com
Carolina Reaper	*C. chinense*	Puckerbutt Seeds, Fort Mill, SC
Brainstrain Yellow	*C. chinense*	Pepperlover.com
Trinidad Scorpion	*C. chinense*	Refining Fire Chiles, San Diego, CA, USA
7-Pot Jonah	*C. chinense*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Trinidad Douglah	*C. chinense*	Refining Fire Chiles, San Diego, CA, USA
Trinidad 7-pot Primo	*C. chinense*	Pepperlover.com
Habanero Orange Blob	*C. chinense*	Dr. Joe Delaney, St. Paul, MN, USA
Trinidad Scorpion Chocolate	*C. chinense*	Dr. Joe Delaney, St. Paul, MN, USA
Tobago Scotch Yellow	*C. chinense*	Dr. Joe Delaney, St. Paul, MN, USA
Trinidad 7-pot	*C. chinense*	Refining Fire Chiles, San Diego, CA, USA
7-pot Brown	*C. chinense*	Pepperlover.com

Chili Pepper Cultivar Name	Species	Seed Source
Trinidad 7-pot Brainstrain Red	*C. chinense*	Pepperlover.com
Trinidad Large 7-pod Yellow	*C. chinense*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Yellow Moruga	*C. chinense*	Pepperlover.com
Congo Trinidad	*C. chinense*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Brown Scotch Bonnet	*C. chinense*	Dr. Joe Delaney, St. Paul, MN, USA
7-pot	*C. chinense*	Refining Fire Chiles, San Diego, CA, USA
Trinidad Moruga Scorpion	*C. chinense*	Chile Pepper Institute, Las Cruces, NM, USA
Bhut Jolokia Red	*C. chinense*	Chile Pepper Institute, Las Cruces, NM, USA
Tobago Scotch Bonnet Red	*C. chinense*	Dr. Joe Delaney, St. Paul, MN, USA
HHP Moruga	*C. chinense*	Hugo Feed Mill and Hardware, Hugo, MN, USA

Chili Pepper Cultivar Name	Species	Seed Source
Brown Trinidad Moruga Scorpion	*C. chinense*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Scotch Bonnet	*C. chinense*	Reimer Seeds, Saint Leonard, MD, USA
Red Majesty Sweet	*C. annuum*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Romanian Rainbow	*C. annuum*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Tasty Paprika GL	*C. annuum*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Red Ruffled Pimiento	*C. annuum*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Orange Blaze	*C. annuum*	Hugo Feed Mill and Hardware, Hugo, MN, USA

L. monocytogenes Scott A, *Salmonella* Typhimurium LT2 and *E. coli* O157:H7 EDL933 bacterial cultures were obtained from the Food Safety Microbiology Lab (Department of Food Science and Nutrition), while the *Staphylococcus aureus* 6538 and *Candida albicans* ATCC 10231 cultures were obtained from the Hegeman Lab (Department of Horticultural Science), both at the University of Minnesota – Twin Cities (St. Paul, MN).

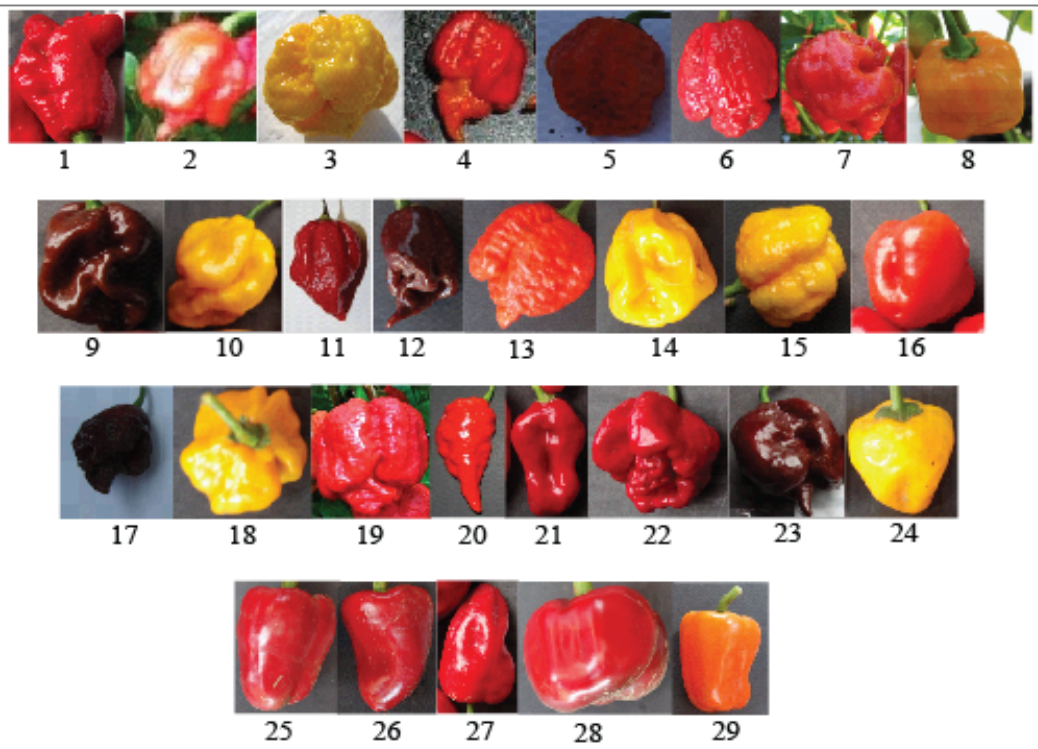
L. monocytogenes was plated on BHI plates, while the other microbes were plated on TSA plates. The plates were then incubated overnight at 37^{o}C and stored at 4^{o}C until use. The test cultures were prepared by inoculating 5 ml of growth media and incubating overnight at 37^{o}C. BHI broth was used for *L. monocytogenes* and TSB was used for all the other microbes.

3.3.2 Methods

3.3.2.1 Chili pepper sample preparation

29 varieties of chili peppers were used: 24 superhot (samples 1 - 24) and five sweet (samples 25 - 29) (Figure 6). For each variety, samples were collected from three different plants and treated independently, giving a total of 87 samples. The peppers samples were prepared by crude methanol extraction. Briefly, a whole pepper was washed under running water and dried off with paper towels. 5 g of whole pepper was then chopped into small pieces and put into a 50 ml conical tube. 8 stainless steel beads were added to the tube (SSB14B stainless steel beads for homogenizer bullet blender, 0.9 - 2.0 mm diameter). The 5 g were then homogenized in a bullet blender (tissue

homogenizer) for 12 min. 12.5 ml of 100% methanol were added into the slurry and homogenized again for 12 min. The liquid portion of the final slurry was pipetted into a centrifuge tube, and centrifuged for 23 min at 4,000 rpm. The supernatant was filter-sterilized through a 0.2 µm Acrodisc filter into a sterile 25 ml bullet blender tube. These steps were repeated until a total of 87 unique methanol extracts were obtained. These extracts were considered the STOCK samples. 1:2 dilutions were made from portions of the STOCKs, to lower the methanol concentrations. These dilutions were then used for the test, while the left over STOCKs were stored until needed. All samples (STOCKs and dilutions) were stored at -20^{o}C until use.



1.7 - Pod Congo SR Gigantic
2.Carolina Reaper
3.Brainstrain Yellow
4.Trinidad Scorpion
5.7 - Pot Jonah
6.Trinidad Douglah
7.Trinidad 7–Pot Primo
8.Habanero Orange Blob
9.Trinidad Scorpion Chocolate
10.Tobago Scotch Yellow
11.Trinidad 7–Pot
12.7–Pot Brown
13.Trinidad 7- Pot Brainstrain Red
14.Trinidad Large 7– Pod Yellow
15.Yellow Moruga
16.Congo Trinidad
17.Brown Scotch Bonnet
18.7–Pot
19.Trinidad Moruga Scorpion
20.Bhut Jolokia Red
21.Tobago Scotch Bonnet Red
22.HHP Moruga
23.Brown Trinidad Moruga Scorpion
24.Scotch Bonnet
25.Red Majesty Sweet
26.Romanian Rainbow
27.Tasty Paprika GL
28.Red Ruffled Pimento
29.Orange Blaze

Figure 6: Pictures of the 29 chili pepper samples used in this study

3.3.2.2 Capsaicin assay

Determining the capsaicin content of chili peppers can be done in different ways. The capsaicin assay kit, from Beacon Analytical Systems was chosen for this study. This method is commonly used, and the standardized kit makes it easier to compare data from different labs (31-33). Liquid Chromatography (LC) (33), and High-Performance Liquid Chromatography (HPLC) (34, 35) are alternative methods. These last two methods are more complex and costly, compared to the assay kit.

The capsaicin content of each of the pepper samples in this study was determined using the Capsaicin Plate Kit (Cat. #20-0027), as per the manufacturer's instructions as follows: The reagents and pepper samples were equilibrated to room temperature. The required number of mixing wells and antibody coated strips were removed from the plastics bags. The bags were resealed to limit exposure to moisture. 100 µl of the calibrators/samples were pipetted into the appropriate mixing wells, using a clean pipette tip for each solution/sample to avoid cross contamination. 100 µl of the enzyme conjugate were added to each well. The contents of the wells were mixed gently by pipetting up and down a few times with a multichannel pipette. 100 µl of the mixture were transferred into the antibody coated reactions wells. The plate was swirled rapidly to mix the contents and covered with Parafilm. The plate was then incubated for 10 min. The mixing well was discarded. After incubation, the contents of the well were discarded into a sink. The wells were flooded completely with cool running tap water, and then shaken to empty them. This wash step was repeated four times for a total of five washes.

The plate was inverted on absorbent paper and tapped to remove as much excess water as possible. 100 µl of substrate was added to each well. The wells were covered again and incubated for 10 minutes. 100 µl of the stop solution was added to each well in the same order of addition as the substrate. The plate was read on a microtiter plate reader at 450 nm, and the capsaicin concentration of the pepper samples determined using a series of equations.

The average OD of each set of calibrators, controls and samples were measured on a ChroMate plate reader (ChroMate Awareness Technology Inc.). The %B/Bo values were calculated as follows:

$$\%\ B/Bo = \frac{\text{(Average OD of calibrator, control or sample x 100)}}{\text{Average OD of negative control}}$$

The Bo refers to the average absorbance of the 0 ppm standard (negative control). The % B/Bo compares the average sample or calibrator absorbance to the absorbance of the negative control.

The %B/Bo of each calibrator was graphed on the Y (linear) axis against its concentration on the X (log) axis in MS Excel. The best-fit line was drawn through the calibrator points. The capsaicin concentration of each sample was determined by finding its %B/Bo value and the corresponding concentration level on the graph, and multiplying that number by the appropriate dilution factor. Calculation of sample concentration is only valid if the %B/Bo of the sample falls within the range of the %B/Bo set by the

calibrators. If the sample falls outside of that range, the results must be reported as less than the lowest calibrator value or greater than the highest calibrator value (36).

3.3.2.3 Resazurin assays

The viability of cell cultures is routinely assessed by utilizing the metabolic capacity of cells which biochemically convert chemicals (usually color dyes). Resazurin (Alamar Blue) is an example of one of these metabolically active compounds. Resazurin is used to non-destructively study cell viability and proliferation. It is simple, sensitive, rapid, robust and reliable, and could be used successfully to assess antibacterial properties of natural products (37-41). In this study, resazurin was used as an indicator of cell viability after exposure to chili pepper extracts, as previously done by Twigg *et al.* (25).

0.02% resazurin dye was prepared, filter-sterilized through a 0.2 µm Acrodisc and stored at 4^{o}C until use. For each test, the pepper samples were pipetted into a sterile 96 micro-well plate. Dilutions were made accordingly, where necessary. The resulting total volume of the sample in each test well was 100 µl. The inoculum was prepared from an overnight culture by adjusting the OD_{600nm} to 0.08 – 0.13 A. 100 µl of the inoculum was pipetted into each test well. An equivalent of the methanol in the samples used was pipetted into the positive control well. The methanol was diluted with broth (BHI for *L. monocytogenes* and TSB for all other microbes) for a total of 100 µl. And finally, 100 µl of the inoculum was added into the well. 100 µl of the respective broth was pipetted into the growth control well. 100 µl of the inoculum was then added into the well. An equivalent of the methanol in the samples used was pipetted into the negative control

well. The methanol was diluted with the respective broth for a total of 200 µl. 20 µl of the resazurin dye was then added into all the wells in use. This set-up is summarized in Table 14. The plates were incubated for 2 h (2 h was determined to be short enough to avoid reagent toxicity but long enough to provide adequate sensitivity). After incubation, the plates were photographed and the results recorded based on comparisons made between the test wells and the controls.

Table 14: The 96-microwell plate set-up of the resazurin and growth inhibition tests done in this study

Test wells	Amount of pepper sample (µl)	Diluent (TSB/BHI) (µl)	Inoculum (OD_{600nm} = 0.02 A) (µl)	Total volume of each well (µl)
1:4	25	75	100	200
1:8	12.5	83.5	100	200
1:16	6.25	93.75	100	200
1:32	3.125	96.875	100	200
Positive control	(100%) methanol			
1:4	25	50	100	200
1:8	12.5	83.5	100	200
1:16	6.25	93.75	100	200
1:32	3.125	96.875	100	200
Negative control	1:2 (100%) methanol			
1:4	25	150	N/A	200
1:8	12.5	183.5	N/A	200
1:16	6.25	193.75	N/A	200
1:32	3.125	196.875	N/A	200
Growth control	N/A	100	100	200

3.4 Results

3.4.1 Capsaicin assay

Figure 7 shows the standard curve obtained from the capsaicin assay. Once the %B/Bo values were calculated as previously stated, the capsaicin content of the chili pepper samples were determined (ppm) from the equation of the best-fit line. The ppm values were then multiplied by 16 to get SHU. The results are presented in Table 15. Figures 8 and 9 summarize the average capsaicin values for each chili pepper variety used in this study.

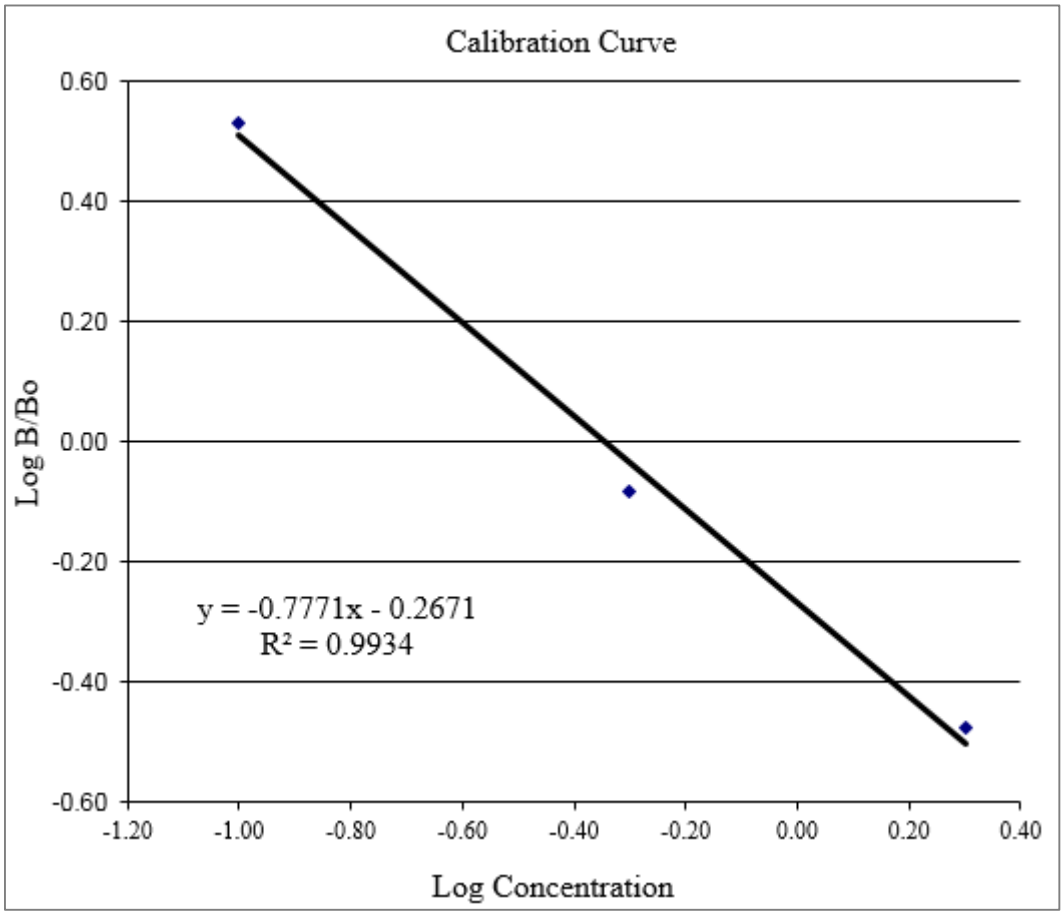


Figure 7: Standard curve from the capsaicin assay

Table 15: Labels, names and capsaicin content of the chili pepper samples in this study determined using Capsaicin Plate Kit (Cat. #20-0027) assay

		Capsaicin content		
Chili pepper name	**Label**	**µg/ml**	**SHU**	**µg/g of pepper**
7 - Pod Congo SR Gigantic	1A	9,438	151,007	1,888
7 - Pod Congo SR Gigantic	1B	3,384	54,143	677
7 - Pod Congo SR Gigantic	1C	8,720	139,522	1,744
Carolina Reaper	2A	944	15,111	189
Carolina Reaper	2B	1,431	22,895	286
Carolina Reaper	2C	5,959	95,339	1,192
Brainstrain Yellow	3A	7,367	117,868	1,473
Brainstrain Yellow	3B	14,974	239,581	2,995
Brainstrain Yellow	3C	177	2,829	35
Trinidad Scorpion	4A	6,616	105,861	1,323
Trinidad Scorpion	4B	16,699	267,182	3,340
Trinidad Scorpion	4C	61,496	983,936	12,299
7 - Pot Jonah	5A	29,188	467,016	5,838
7 - Pot Jonah	5B	34,068	545,090	6,814
7 - Pot Jonah	5C	34,350	549,603	6,870
Trinidad Douglah	6A	44,135	706,156	8,827
Trinidad Douglah	6B	17,369	277,898	3,474

Table continued on the next page

Chili pepper name	Capsaicin content			
	Label	µg/ml	SHU	µg/g of pepper
Trinidad Douglah	6C	27,401	438,415	5,480
Trinidad 7-pot Primo	7A	7,160	114,552	1,432
Trinidad 7-pot Primo	7B	58,079	929,268	11,616
Trinidad 7-pot Primo	7C	50,706	811,293	10,141
Habanero Orange Blob	8A	6,469	103,498	1,294
Habanero Orange Blob	8B	2,839	45,420	568
Habanero Orange Blob	8C	4,777	76,433	955
Trinidad Scorpion Chocolate	9A	9,172	146,755	1,834
Trinidad Scorpion Chocolate	9B	9,455	151,287	1,891
Trinidad Scorpion Chocolate	9C	8,207	131,319	1,641
Tobago Scotch Yellow	10A	7,259	116,146	1,452
Tobago Scotch Yellow	10B	6,601	105,620	1,320
Tobago Scotch Yellow	10C	16,076	257,221	3,215
Trinidad 7 - Pot	11A	19,544	312,701	3,909
Trinidad 7 - Pot	11B	59,055	944,887	11,811
Trinidad 7 - Pot	11C	37,131	594,095	7,426
7 - Pot Brown	12A	8,879	142,071	1,776
7 - Pot Brown	12B	33,427	534,831	6.685

Table continued on the next page

Chili pepper name	Label	Capsaicin content		
		µg/ml	SHU	µg/g of pepper
7 - Pot Brown	12C	15,893	254,283	3,179
Trinidad 7- Pot Brainstrain Red	13A	33,677	538,826	5,153
Trinidad 7- Pot Brainstrain Red	13B	68,308	1,092,925	13,662
Trinidad 7- Pot Brainstrain Red	13C	25,916	414,656	6,735
Trinidad Large 7 - Pod Yellow	14A	11,103	177,650	2,221
Trinidad Large 7- Pod Yellow	14B	9,457	151,310	1,891
Trinidad Large 7 - Pod Yellow	14C	13,534	216,543	2,707
Yellow Moruga	15A	9,332	149,313	1,866
Yellow Moruga	15B	10,870	173,912	2,174
Yellow Moruga	15C	12,025	192,405	2,405
Congo Trinidad	16A	5,069	81,101	1,014
Congo Trinidad	16B	2,930	46,880	586
Congo Trinidad	16C	3,341	53,457	668
Brown Scotch Bonnet	17A	8,907	142,512	1,781
Brown Scotch Bonnet	17B	33	533	7
Brown Scotch Bonnet	17C	16,128	258,051	3,226
7 – Pot	18A	27,765	444,242	5,553
7 – Pot	18B	22,178	354,844	4,436

Table continued on the next page

Chili pepper name	Label	Capsaicin content		
		µg/ml	SHU	µg/g of pepper
7 – Pot	18C	20,057	320,909	4,011
Trinidad Moruga Scorpion	19A	40,334	645,340	8,067
Trinidad Moruga Scorpion	19B	45,899	734,385	9,180
Trinidad Moruga Scorpion	19C	35,557	568,906	7,111
Bhut Jolokia Red	20A	45,789	732,627	9,158
Bhut Jolokia Red	20B	27,190	435,032	5,438
Bhut Jolokia Red	20C	26,747	427,959	5,349
Tobago Scotch Bonnet Red	21A	57	915	11
Tobago Scotch Bonnet Red	21B	15	243	3
Tobago Scotch Bonnet Red	21C	16	251	3
HHP Moruga	22A	35,411	566,578	7,082
HHP Moruga	22B	34,307	548,908	6,861
HHP Moruga	22C	34,706	555,303	6,941
Brown Trinidad Moruga Scorpion	23A	26,441	423,057	5,288
Brown Trinidad Moruga Scorpion	23B	28,408	454,524	5,682
Brown Trinidad Moruga Scorpion	23C	15,189	243,028	3,038
Scotch Bonnet	24A	2,012	32,195	402
Scotch Bonnet	24B	2,350	37,593	470

Table continued on the next page

Chili pepper name	Label	Capsaicin content		
		µg/ml	SHU	µg/g of pepper
Scotch Bonnet	24C	849	13,577	170
Red Majesty Sweet	25A	16	251	3
Red Majesty Sweet	25B	16	261	3
Red Majesty Sweet	25C	13	211	3
Romanian Rainbow	26A	15	241	3
Romanian Rainbow	26B	13	212	3
Romanian Rainbow	26C	15	239	3
Tasty Paprika GL	27A	8	132	2
Tasty Paprika GL	27B	9	148	2
Tasty Paprika GL	27C	9	137	2
Red Ruffled Pimento	28A	11	171	2
Red Ruffled Pimento	28B	12	190	2
Red Ruffled Pimento	28C	12	188	2
Orange Blaze	29A	14	221	3
Orange Blaze	29B	11	175	2
Orange Blaze	29C	13	212	3

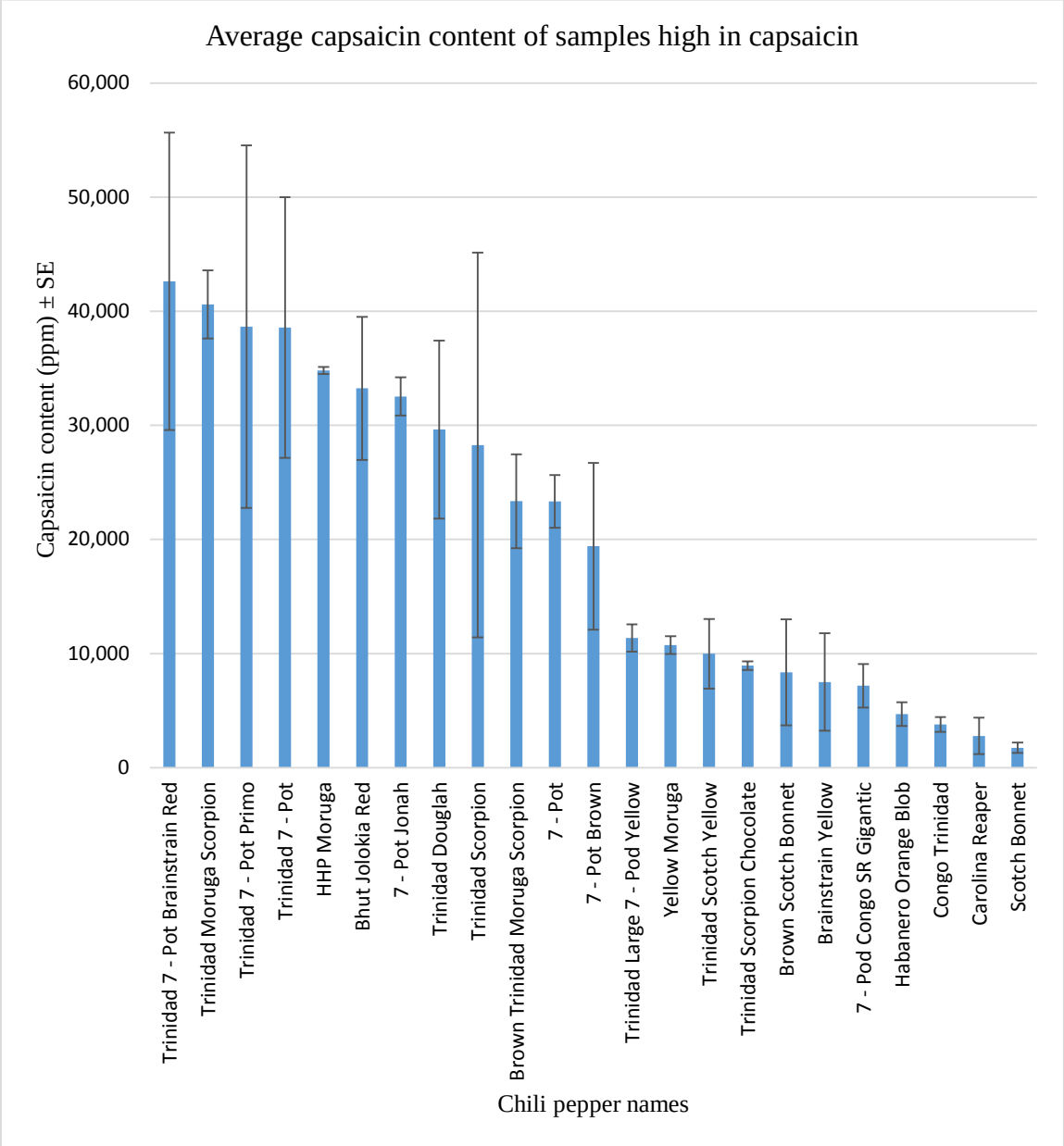


Figure 8: Average capsaicin content of the chili pepper samples high in capsaicin

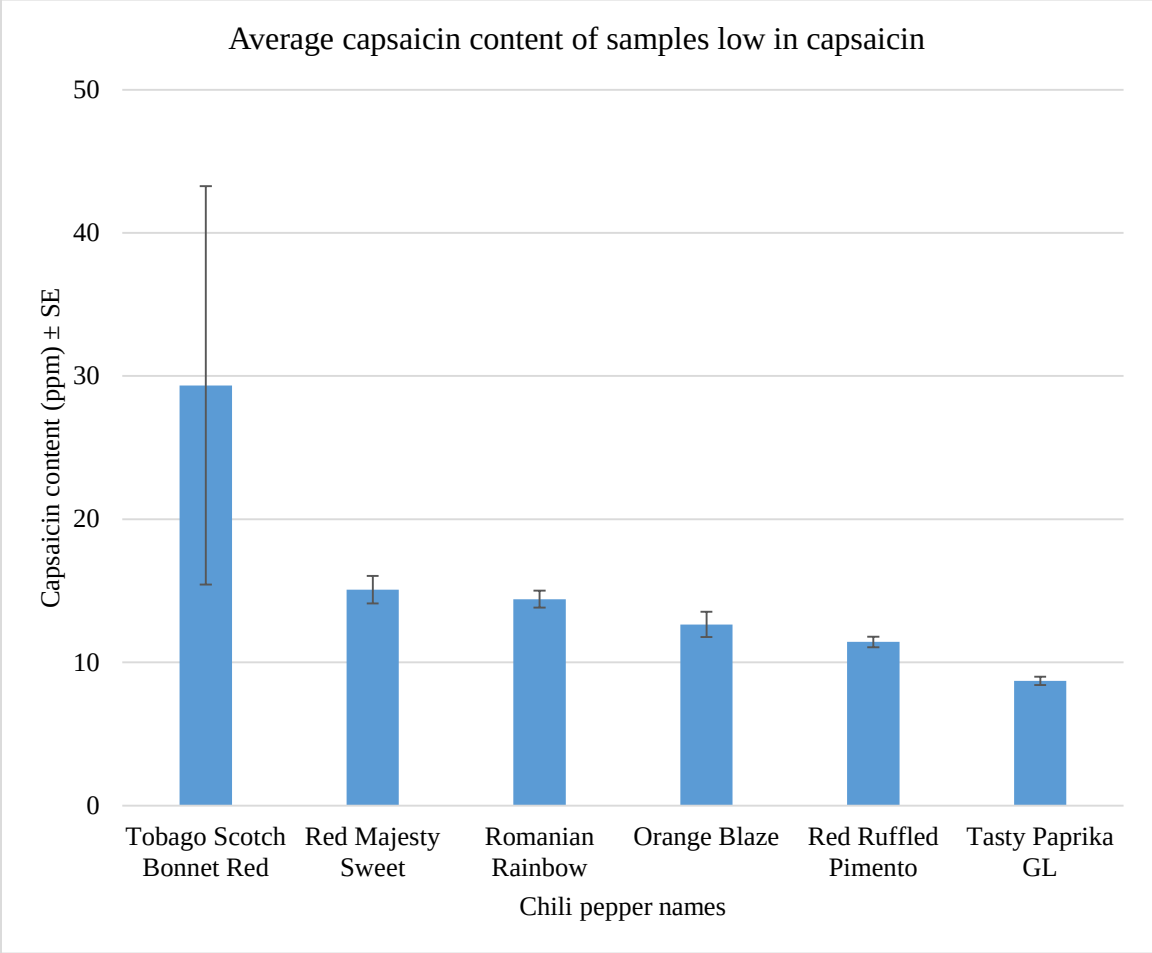


Figure 9: Average capsaicin content of the chili pepper samples low in capsaicin

3.4.2 Resazurin assays

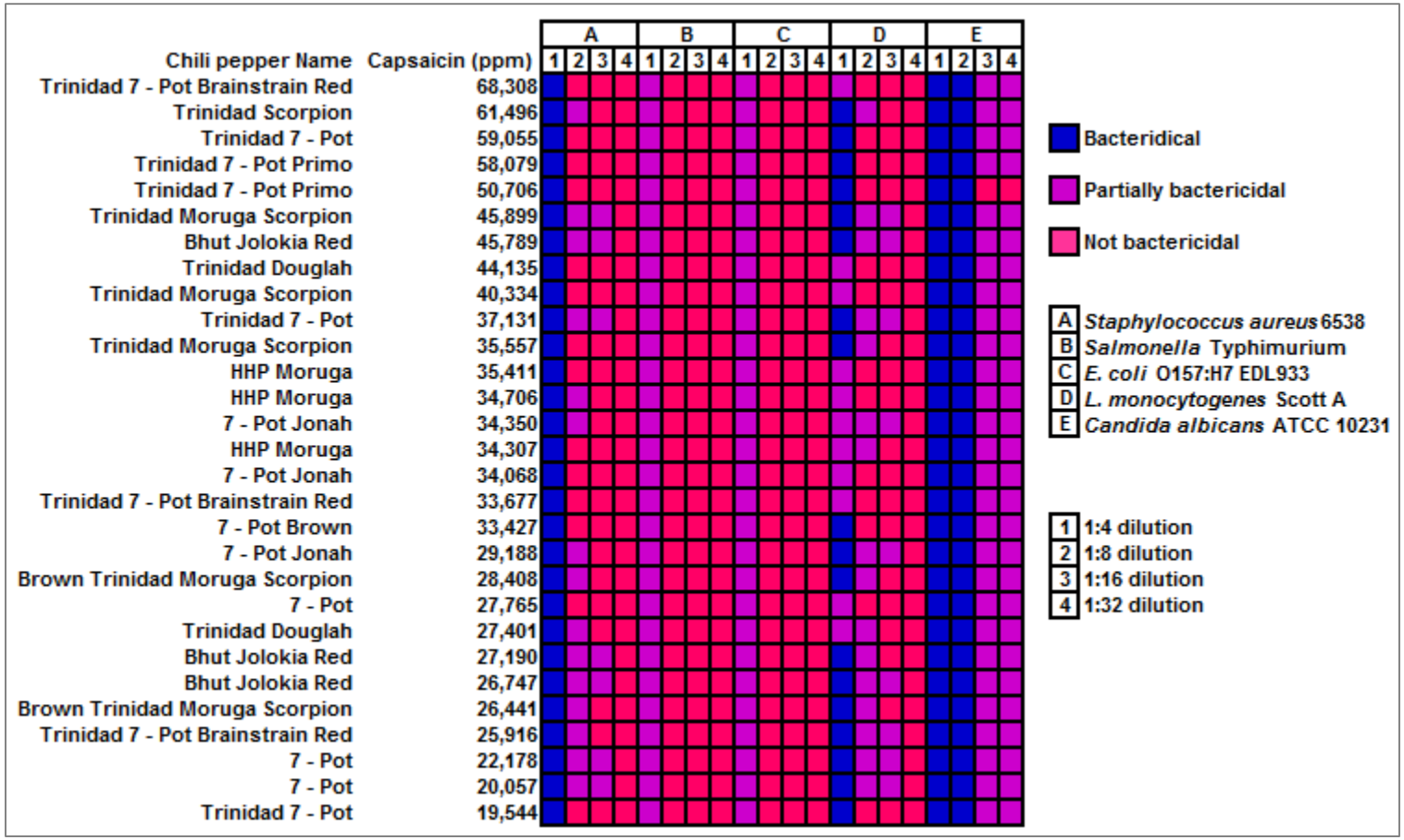
Data from the resazurin assay is presented in Figure 10. An initial negative control test was conducted to ensure that the chili pepper extracts alone would not have an effect on the resazurin dye color after the 2 h incubation period. This addressed the issue of interference from other potentially redox active substances in the peppers themselves. The negative control in the test wells was a measure for contamination of the diluent used in well preparations. The positive control ensured that the methanol concentration in the samples did not affect cell viability. The growth control ensured that the cells cultured were viable to begin with.

A dark blue color meant that no viable cells were present in the well after the 2 h incubation period (the chili pepper samples were bactericidal). If the color changed to pink, then viable cells were present (the chili pepper samples had no effect of viability). The purple color signified that the concentration of chili pepper sample used was not high enough to kill all the microbial cells (the chili pepper samples were partially bactericidal).

Of the samples with low capsaicin content, only Tobago Scotch Bonnet Red showed an effect on microbial viability (partially bactericidal against the Gram positive bacteria, and the yeast). For the samples high in capsaicin, there was a dose dependent antimicrobial response. These effects varied between chili pepper types as indicated in Figure 10.

Candida albicans showed the greatest susceptibility to the chili pepper samples, with effects noted at dilutions as high as 1:32. At the 1:4 and 1:8 dilution, the methanol

concentrations in all the samples were high enough to kill the yeast cells, as indicated by the positive control. So, the 1:4 and the 1:8 dilution data is presented only for completion. *Salmonella* Typhimurium and *E. coli* O157:H7 were least susceptible. For *Staphylococcus aureus*, two chili peppers showed effects at the 1:16 dilution (Bhut Jolokia and 7-Pot). *L. monocytogenes* was affected by three samples at the 1:16 dilution (Bhut Jolokia, 7-Pot Jonah and Brown Scotch Bonnet) (Figure 10).



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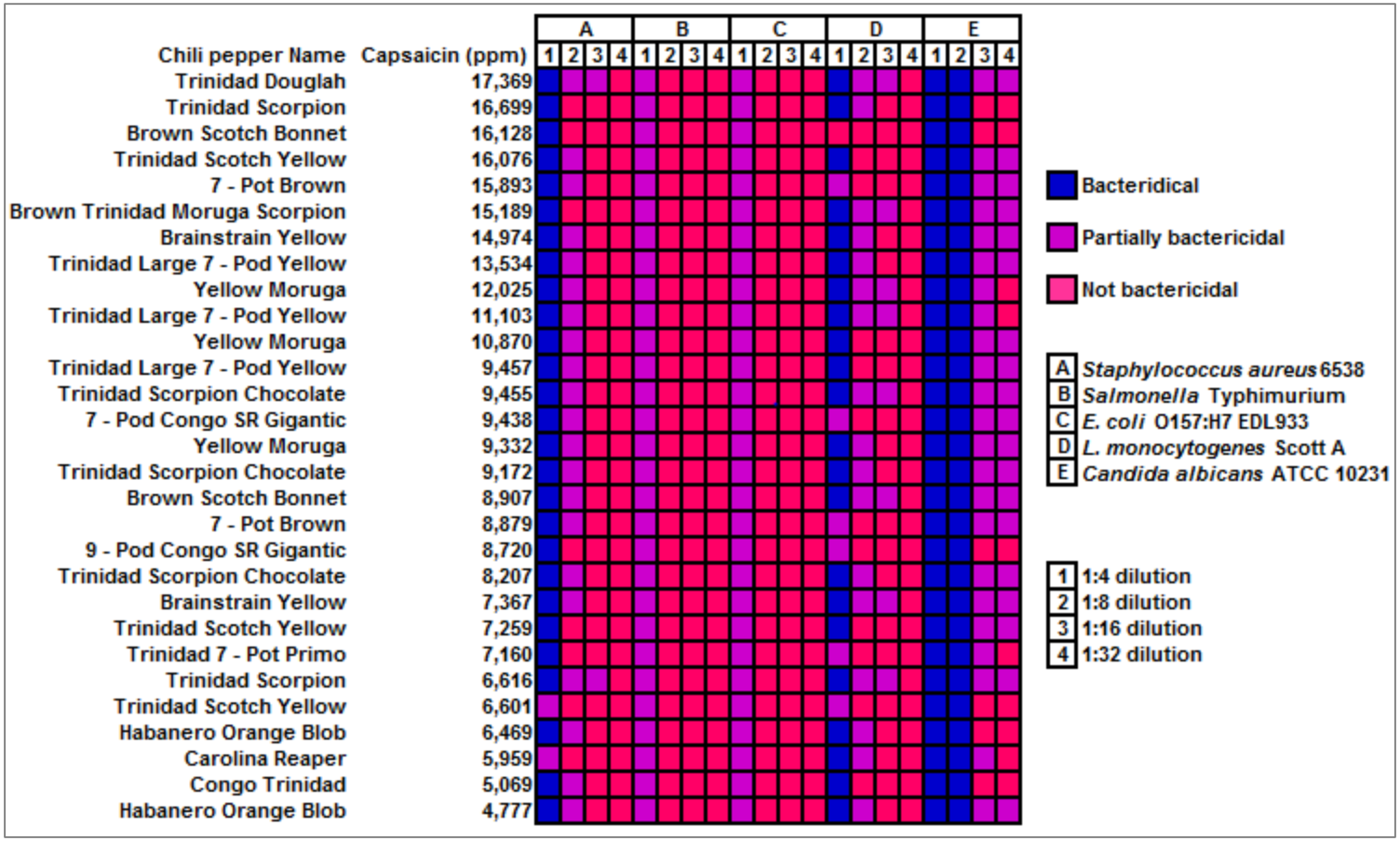

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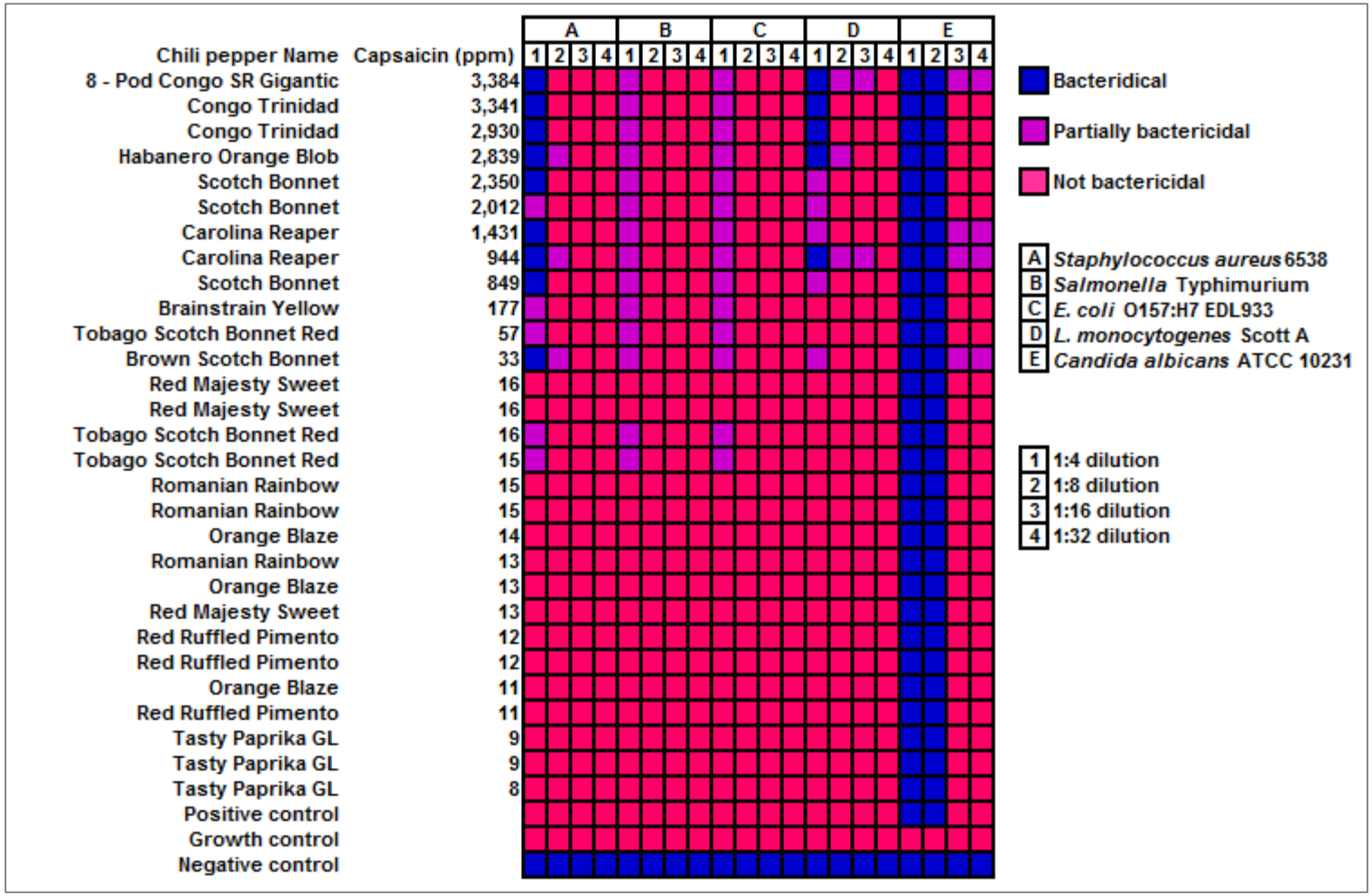

Figure 10: Effects of the chili pepper extracts on microbial viability

3.5 Discussion

3.5.1 Capsaicin assay

The Beacon Capsaicin Plate Kit assay is a polyclonal antibody assay specific for capsaicin with reactivity to a limited number of closely related compounds (e.g. other capsaicinoids). Table 16 shows the relative values for 50% Bo and the percent cross-reactivity (%CR) versus capsaicin (natural). All concentrations are in parts per million (ppm) (36).

Capsaicin is the most abundant capsaicinoid in chili peppers. The others include dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin (their structural differences were previously listed in Table 1). It has been established that, less spicy varieties have capsaicinoids concentration range 0.003% - 0.01%. Mildly hot varieties have capsaicinoids range from 0.01% - 0.3%, while the hot spicy ones have concentration range is 0.3% - 1% capsaicinoids of the total dry weight (42). It is not clear whether the minor capsaicinoids are accounted for in the capsaicin assay results because they have not been tested for. There are five levels of pungency classified using Scoville heat units: non-pungent (0 – 700 SHU), mildly pungent (700 – 3,000 SHU), moderately pungent (3,000 – 25,000 SHU), highly pungent (25,000 – 70,000 SHU) and very highly pungent (>80,000 SHU) (35).

The amount of capsaicinoids in a chili pepper pod is dependent on the genetic makeup of the plant, the environment (light intensity, temperature, etc.) in which the

plant is grown, the age of the fruit, and the position of the fruit on the plant. The heat level can vary significantly among plants of the same variety grown in a single field at the same time (35). When single plant heat levels of genetically identical chili pepper plants were compared with the field average, it was found that individual plants had heat levels as much as 78% higher than the field average, indicating that the environment contributes significantly to chili pepper heat levels (30). Similar variations were observed in the data obtained from the capsaicin assay in this study (Figures 8 and 9).

Capsaicinoid biosynthesis is restricted to the genus *Capsicum* and results from the acylation of the aromatic compound, vanillylamine, with a branched-chain fatty acid. The presence of capsaicinoids is controlled by the *Pun1* locus, which encodes a putative acyltransferase. In its homozygous recessive state, *pun1/pun1*, capsaicinoids are not produced by the pepper plant (31). The trace amounts of capsaicin in the "sweet" chili peppers were most likely because ancestral chili peppers all contained capsaicin, and the "sweet" chili varieties can revert back to this ancestral trait. The capsaicin data obtained in this study was referenced to determine whether or not there is a correlation between capsaicin content and the effects of the chili pepper extracts on microbial viability and growth.

Table 16: Relative values for 50% Bo and the percent cross-reactivity (%CR) versus capsaicin (natural)

Compound	50% Bo*	%CR
Capsaicin (natural mixture)**	0.625	100
Capsaicin (pure)	0.599	104
Dihydrocapsaicin	0.639	98

*%Bo equals average sample absorbance divided by average negative control absorbance times 100%

**Contains ~ 65 % capsaicin and 35 % dihydrocapsaicin

3.5.2 Resazurin assays

While previous research referenced in the literature review section suggests that capsaicin is the primary antimicrobial in chili peppers, data from this study imply that there might be other compounds with similar properties. Looking at the samples high in capsaicin (Figures 8), suggests that there is no direct correlation between the amounts of capsaicin between chili pepper types and their antimicrobial activity. Instead, there are instances. This idea was also proposed by Dorantes *et al*. (6) and Nascimento *et al*. (43) in their growth inhibition studies.

Although Tobago Scotch Bonnet Red had very little capsaicin compared to the other samples in the *C. chinense* species, it showed antimicrobial effects. This finding was unique because the other samples that had little capsaicin, but belonged to the *C. annuum* species, showed no antimicrobial properties.

Staphylococcus aureus was tested against 3 different dilutions of the pepper samples (1:4, 1:8 and 1:16). At the 1:4 dilution, the bacterial cells were killed by 22 out of the 24 “hot” samples. When the samples were diluted 2 - fold, viability was affected by 13 of the “hot” samples, but there was no bactericidal effect. Surprisingly, the 5 hottest samples had no effect at the 1:8 dilution. After a 4 - fold dilution, only 2 of the samples still affect viability (Bhut Jolokia and 7-Pot) (Figure 10).

None of the samples (at the concentrations tested), killed *Salmonella* Typhimurium. Instead, the resazurin dye changes color to purple (at the 1:4 dilution), indicating that the cells are not dead, but are spending energy trying to overcome the

stress from the samples and less on metabolism. A similar effect was noted for *E. coli* O157:H7 (Figure 10).

Against *L. monocytogenes,* 15 samples killed the cells at the 1:4 dilution. The remaining 9 samples affected viability, but were not bactericidal. At a 1:8 dilution, 22 of the 24 samples affected viability, as indicted by the purple color. At 1:16 dilution, only Bhut Jolokia, 7-Pot Jonah and Brown Scotch Bonnet showed some effect on viability (Figure 10). *Candida albicans* showed most susceptibility to the samples. Samples diluted as much as 1:32 still affected the viability of the cells.

Based on these findings, it was concluded that *E.coli* 0157:H7 and *Salmonella* Typhimurium less susceptible to the chili pepper samples. Both of these bacteria are Gram negative (contain an additional outer membrane) and are generally more resistant to antimicrobials (44). For a fungi that is commensal to the human gut, it is surprising that *Candida albicans* has not developed a resistance to capsaicin. This brings to question the mechanism by which capsaicin affects fungal cells. Kurita *et al*., in their studies on the antimicrobial mechanisms of capsaicin using yeast DNA microarray, suggest that capsaicin enters the cells and functions as a toxin, possibly to the membrane structure and/or as osmotic stress (45). As previously mentioned, *Candida albicans* is commensal to the human gut, and helps to maintain a healthy microbiome. This study suggests that consuming high levels of chili peppers may tip that balance. However, an *in vivo* study would be necessary to make any conclusive assumptions. It was determined that 200 ppm

of 99.1% pure capsaicin needed to obtain observable antibacterial effect on the microorganisms tested (resazurin color ceased to change to purple/pink).

4 Effects of chili pepper samples on microbial growth

4.1 Summary

In the food industry, control of microbial growth is an essential part of ensuring food safety. Food producers use preservatives to extend the shelf life of food. However, some spices offer the potential for natural antimicrobials. Growth inhibition assays allow scientists to determine the effectiveness of antimicrobials at different concentrations. Generally, the lower the inhibitory concentration, the more effective the antimicrobial. In this study, the growth of microbes exposed to chili pepper samples was monitored on a microplate reader over 18 h. The microplate reader technique is particularly suited for determination of growth inhibition curves, since it is fast, reliable, and requires small total culture volume. For the chili pepper samples high in capsaicin, a dose dependent response was observed. None of the samples low in capsaicin showed any inhibitory effects.

4.2 Introduction

Microorganisms are living entities of microscopic size and include bacteria, viruses, yeasts and mold (together designated as fungi), algae and protozoa. They are present everywhere on earth, which includes in and on humans, animals, plants and other living creatures, soil, water and atmosphere. They multiply everywhere except in the atmosphere. Together their numbers exceed all other living cells on earth (46).

With time, the importance of microorganisms in human and animal disease, soil fertility, plant disease, fermentation, food spoilage and foodborne diseases, and other areas was recognized, and microbiology was developed as a separate discipline. Later, it was divided into several disciplines, such as medical microbiology, plant pathology and food microbiology. Although viruses are known to cause foodborne illnesses, food microbiologists have more information on bacteria, which are implicated in a majority of outbreaks and food recalls. Fungi generally cause food spoilage (46, 47).

Bacteria are classified as strict aerobes (need oxygen to survive), strict anaerobes (are killed by the presence of oxygen) or facultative anaerobes (can live with or without oxygen). Like all living cells, each bacterium requires food for energy and building materials. Although they are countless on Earth, most are harmless, and many are even beneficial to humans. In fact, less than 1 percent of bacteria cause diseases in humans. For example, harmless anaerobic bacteria, such as *Lactobacilli acidophilus*, live in our intestines, where they help digest food, destroy disease-causing microbes, fight cancer cells, and give the body needed vitamins. Healthy food products, such as yogurt, sauerkraut, and cheese, are made using bacteria (47).

However, there are bacteria that are harmful to us. Those that are transmitted through food are collectively called foodborne pathogens. A foodborne illness (sometimes called “foodborne disease”, “foodborne infection”, or “food poisoning”) is a common, costly—yet preventable—public health problem. Each year, one in six Americans gets sick by consuming contaminated foods or beverages. Many different

disease-causing microbes, or pathogens can contaminate foods, so there are many different foodborne infections. A few of the commonly known foodborne pathogens include *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes, Staphylococcus aureus* and *Clostridium botulinum*. They enter our food from many sources such as contaminated water, food handlers with poor personal hygiene and from contaminated food processing equipment (48).

There are many ways to ensure that food remains uncontaminated until it reaches consumers. Good manufacturing practice (GMP) is an example. GMP is a system to ensure that products meet food safety, quality and legal requirements. All food manufacturers should have GMP in place. In addition to foodborne pathogens, food manufacturers must beware of allergens. To address these issues sufficiently, Hazard Analysis and Critical Control Point (HACCP) is the recommended approach. If both GMP and HACCP are followed religiously, consumers should receive safe food on the shelves (49).

The control of microbial growth is necessary in many practical situations. Significant advances in agriculture, medicine and food science have been made through study of this area of microbiology. Inhibiting or controlling the growth of bacteria involves the use of physical or chemical agents. These agents are either cidal (kill the bacteria) or static (inhibit growth without killing the bacteria). Early civilizations practiced salting, smoking, pickling and drying to control microbial growth. Some spices

prevented the spoilage of food, but were mainly used to mask the taste of spoiled food (4).

It was not until the 1800's when Joseph Lister established the concept of aseptic techniques. He based his concept on Louis Pasteur's germ theory of disease. These techniques were immediately incorporated in the medical field. Many patients, who would have otherwise died from infections recovered well, leading to a revolution in medical practice (50). The food industry followed soon after. Processing companies incorporated kill steps in the production lines to keep food safe and prolong shelf life. Some of the most common techniques are commercial sterilization (used to denature *Clostridium botulinum* spores), blanching, boiling, canning and pasteurization. Lowering product pH is another way of controlling growth of *Clostridium botulinum* spores. These steps improve the safety and quality of food by reducing the initial microbial load of food products before they are put out on the shelves in grocery stores (46).

The quality and safety of food products are the two factors that most influence the choices made by today's increasingly demanding consumers. Conventional food sterilization and preservation methods often result in a reduction in the apparent freshness and quality of the final products (51). The few residual bacterial cells eventually multiply to levels that could be deleterious to the consumer. The rate at which this occurs depends on the product handling after the kill step. Time temperature combinations are important in maintaining the safety of food. Exposing food to temperature abuse at any point from the processing plant to the fridge at home leads to rapid deterioration. Manufacturers add

preservatives to food products to mitigate the short shelf life of most foods. Preservatives are generally not added to a majority of fresh fruits and vegetables, all of which are highly perishable (52) .

As mentioned previously, early civilizations never knowingly employed "scientific" methods to control microbial growth. However, the methods they used had scientific basis as we know today. Salting and drying both reduce water activity (reducing available water in food) for microbial growth. Spices are chemicals, which affect cells membranes and metabolic processes, much like the preservatives we use today. Mayans are well known for their use of spices (4). Of specific interest in this study, is their use of chili peppers.

The growth inhibition assay is performed to investigate the long term effects (18 h) of the pepper samples on microbial growth. The micro-well plate set-up is similar to that of the resazurin assay, except no dye is needed. A complete growth curve, for most bacteria, typically takes 24 h to obtain. The curve has 4 distinct phases: the lag, log, stationary and death phases (53). The death phase starts to appear after about 18 h of incubation, hence the duration chosen for this test. From the growth curve, it is possible to observe the behavior of the cells over a long period of time, when exposed to the pepper sample.

Bacteriostatic effects are possible. Additionally, the stress exerted by the chili pepper samples could also trigger the cells into entering the VBNC state. Cells that are under this effect could still give a positive test in the resazurin test because cells that are

not growing still need to perform house-keeping metabolic reactions, giving off products that would be redox active. These cells would not show an increase in numbers on the growth curve, unless they overcome the stress (54). This cell recovery concept would be useful if chili peppers were added to food as a preservative. If cells recover from the injuries exerted by the peppers, food would pose a potential danger to consumers, especially because the target products (meats and cheeses) are usually on the shelves for more than 18 h.

In this study, four bacteria and one yeast were tested. *Escherichia coli* O157:H7 is commonly known for its prevalence in ground beef. It is a Gram negative, facultative anaerobe. *Salmonella* Typhimurium is a rod shaped Gram negative, facultative anaerobe, isolated from a variety of foods including peanut butter and chicken. *Listeria monocytogenes* is particularly of concern because of its ability to induce spontaneous abortions and still births in infected expectant women. It is a Gram positive, facultative anaerobe commonly associated with dairy products and ready to eat deli meats. *Staphylococcus aureus* is Gram positive and individual cells commune into grape-like clusters. Foods such as pasta, salads and tacos have been implicated in *Staphylococcus aureus* related outbreaks (48). *Candida albicans* is a yeast commensal to our gut, genitourinary tract and skin. It becomes an opportunistic pathogen under a number of different host conditions, usually involving reduced immune competence or an imbalance of the competing bacterial microflora (55).

4.3 Materials and Methods

4.3.1 Materials

The chili peppers fruits used (Table 13), were cultivated in the University of Minnesota Agricultural fields (St. Paul campus) following the methods of Bosland *et al.* (30), harvested and frozen until samples were prepared.

L. monocytogenes Scott A, *Salmonella* Typhimurium LT2 and *E. coli* O157:H7 EDL933 bacterial cultures were obtained from the Food Safety Microbiology Lab (Department of Food Science and Nutrition), while the *Staphylococcus aureus* 6538 and *Candida albicans* ATCC 10231 cultures were obtained from the Hegeman Lab (Department of Horticultural Science), both at the University of Minnesota – Twin Cities (St. Paul, MN).

The BBL™ Tryptic Soy Agar (TSA), Becto™ Tryptic Soy Broth (TSB) and BBL™ Brain Heart Infusion (BHI) broth were purchased from Becton, Dickinson and Company (Sparks, MD). *L. monocytogenes* was plated on BHI plates, while the other microbes were plated on TSA plates. The plates were then incubated overnight at 37^{o}C and stored at 4^{o}C until use. The test cultures were prepared by inoculating 5 ml of growth media and incubating overnight at 37^{0}C. BHI broth was used for *L. monocytogenes* and TSB was used for all the other microbes.

4.3.2 Methods

4.3.2.1 Growth inhibition assays

For each test, the required volumes of chili pepper samples were pipetted into a sterile 96 micro-well plate. The resulting total volume of the sample in each well was 100 µl. The inoculum was prepared from an overnight culture by adjusting the OD_{600nm} to 0.02 A. 100 µl of the inoculum was pipetted into each test well. An equivalent of the methanol in the samples used was pipetted into the positive control well. The methanol was diluted with (BHI for *L. monocytogenes* and TSB for all other microbes) for a total of 100 µl. And finally, 100 µl of the inoculum was added into the well. 100 µl of the respective broth was pipetted into the growth control well. 100 µl of the inoculum was then added into the well. An equivalent of the methanol in the samples used was pipetted into the negative control well. The methanol was diluted with the respective broth for a total of 200 µl. This set-up is summarized in Table 14. The plates were incubated in a BioTek Epoch 2 microplate reader at 37^{o}C for 18 h. The reader was set to a continuous bi-orbital shake, and readings were taken every 10 min over the 18 h. After incubation, the data obtained (absorbance), was exported into an Excel spreadsheet. The data was plotted to obtain line graphs (growth curves). The shape and height of graphs obtained from the test wells were compared to the data from the 3 controls (positive, negative and growth). From this comparison, the effect of the chili pepper extracts on bacterial growth was determined. A 2- tail student t-test (two-sample assuming unequal variances) was used in determining statistically significant differences between the data obtained from

the samples and the three controls used. A p-value > 0.05 indicated no statistically significant difference, while a p-value < 0.05 indicated statistically significant difference.

Similar tests were done using pure capsaicin (99.1% purity), *m*-coumaric acid and 50% natural capsaicin in place of the chili pepper samples.

4.4 Results

4.4.1 Chili pepper samples

Table 15 lists the labels of the chili pepper samples and what they stand for. These labels were used to allow for unbiased testing. For all the tests, the controls are labelled H8 for the positive, H10 for the growth and H12 for the negative. The summary of the results are presented in Figure 11. The red color indicates no microbial growth (no statistically significant difference between OD_{600nm} of the sample and that of the negative control at the end of the test, $p > 0.05$). The green color indicates normal growth (OD_{600nm} at the end of the test is equal to the OD_{600nm} of the positive control, $p < 0.05$). The orange color indicates partial inhibition (OD_{600nm} at the end of the test is less than the OD_{600nm} of the positive control, $p < 0.05$). None of the “sweet” chili pepper samples were bacteriostatic, thus, the remaining part of the discussion only covers samples with heat values > 250 SHU (total of 72 samples).

64 of the 72 samples diluted 1:4 inhibited the growth of *Staphylococcus aureus*. The remaining eight showed partial inhibition. At the 1:8 dilution, 36 were bacteriostatic.

When the samples were diluted 1:16, 21 samples resulted partial inhibition of growth. Further dilutions showed no effect on growth (Figure 11).

None of the samples diluted 1:4 inhibited the growth of *E. coli* O157:H7 or *Salmonella* Typhimurium. Instead, all the samples partially inhibited growth. Further dilutions had no notable effect on the growth of either bacterium (Figure 11).

57 of the 72 samples inhibited the growth of *L. monocytogenes* at the 1:4 dilution, while 10 partially inhibited growth. At the 1:8 the dilution, 32 of the samples were bacteriostatic. No sample was inhibited growth at the 1:16 dilution and at the 1:32 dilutions, 11 samples of six chili pepper varieties still partially inhibited growth (Trinidad 7-pot Brainstrain Red, Trinidad 7-pot Primo, Bhut Jolokia Red, Trinidad Scorpion, Brown Trinidad Moruga Scorpion, and Brainstrain Yellow). *Candida albicans* was the most susceptible, with 13 samples inhibiting growth at the 1:32 dilution. At the 1:4 and 1:8 dilution, the methanol concentration in all the samples were high enough to kill the yeast cells, as indicated by the positive control, so the data in these columns are presented only for completion (Figure 11).

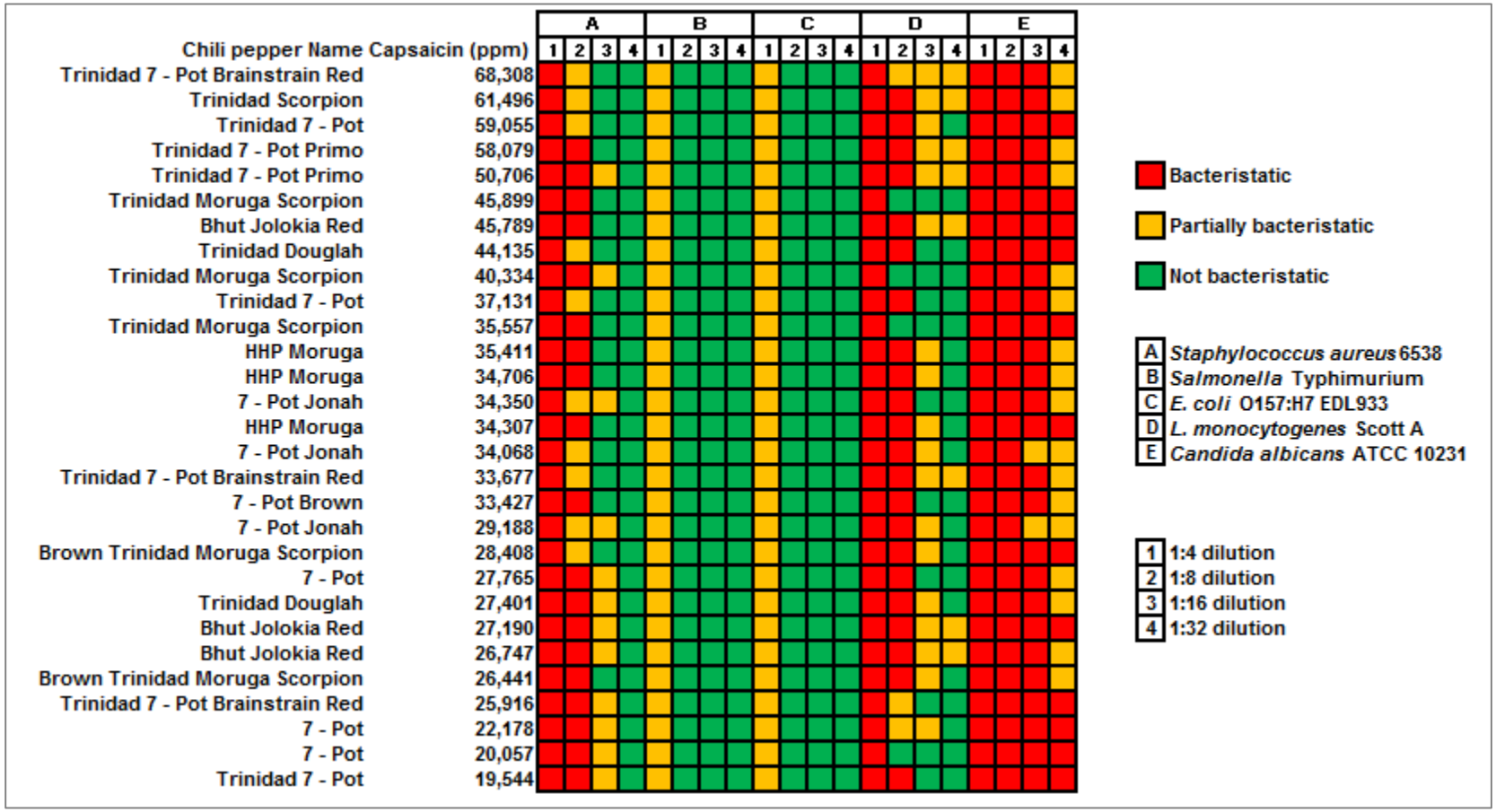

Figure continued on the next page

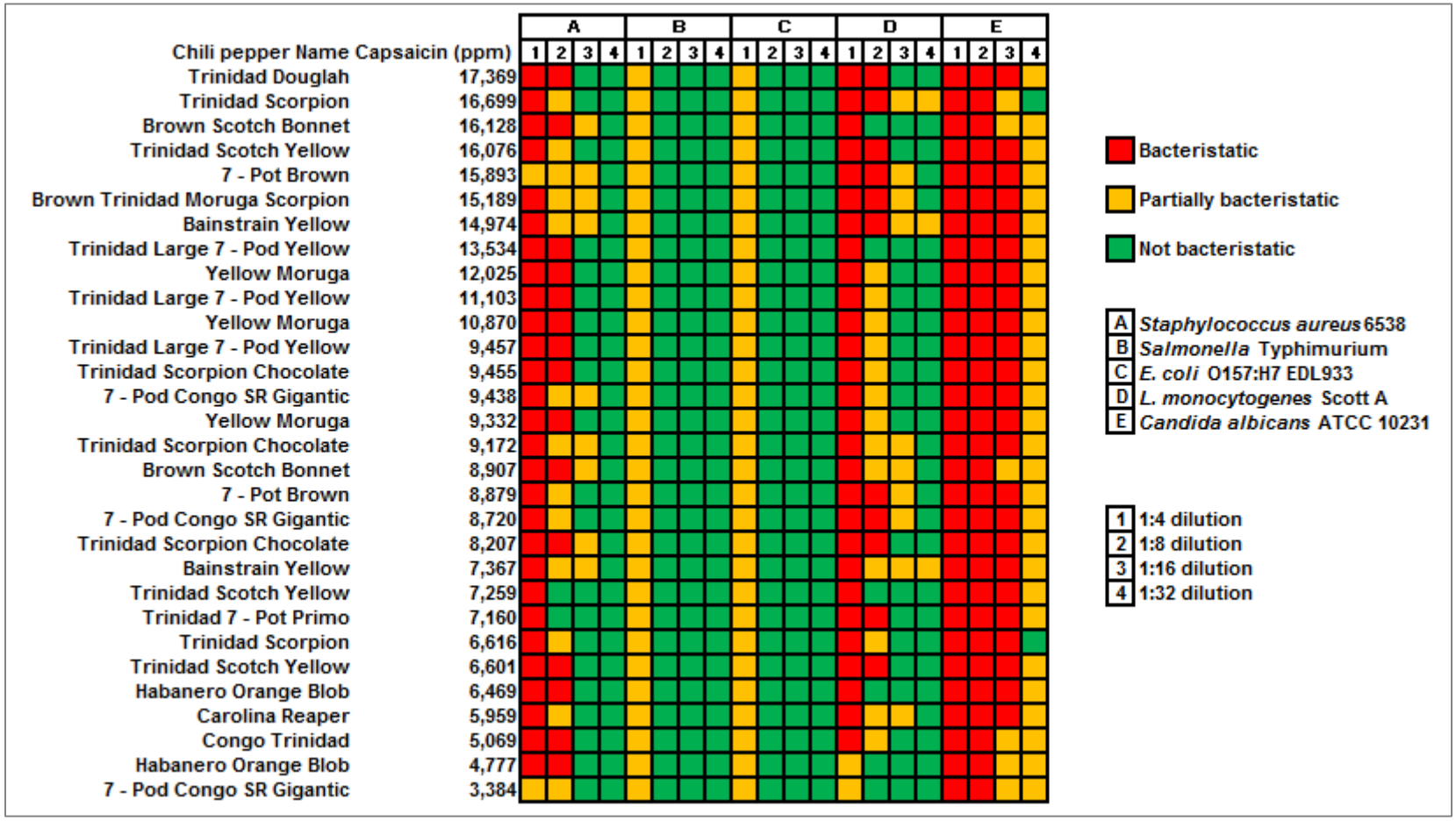

Figure continued on the next page

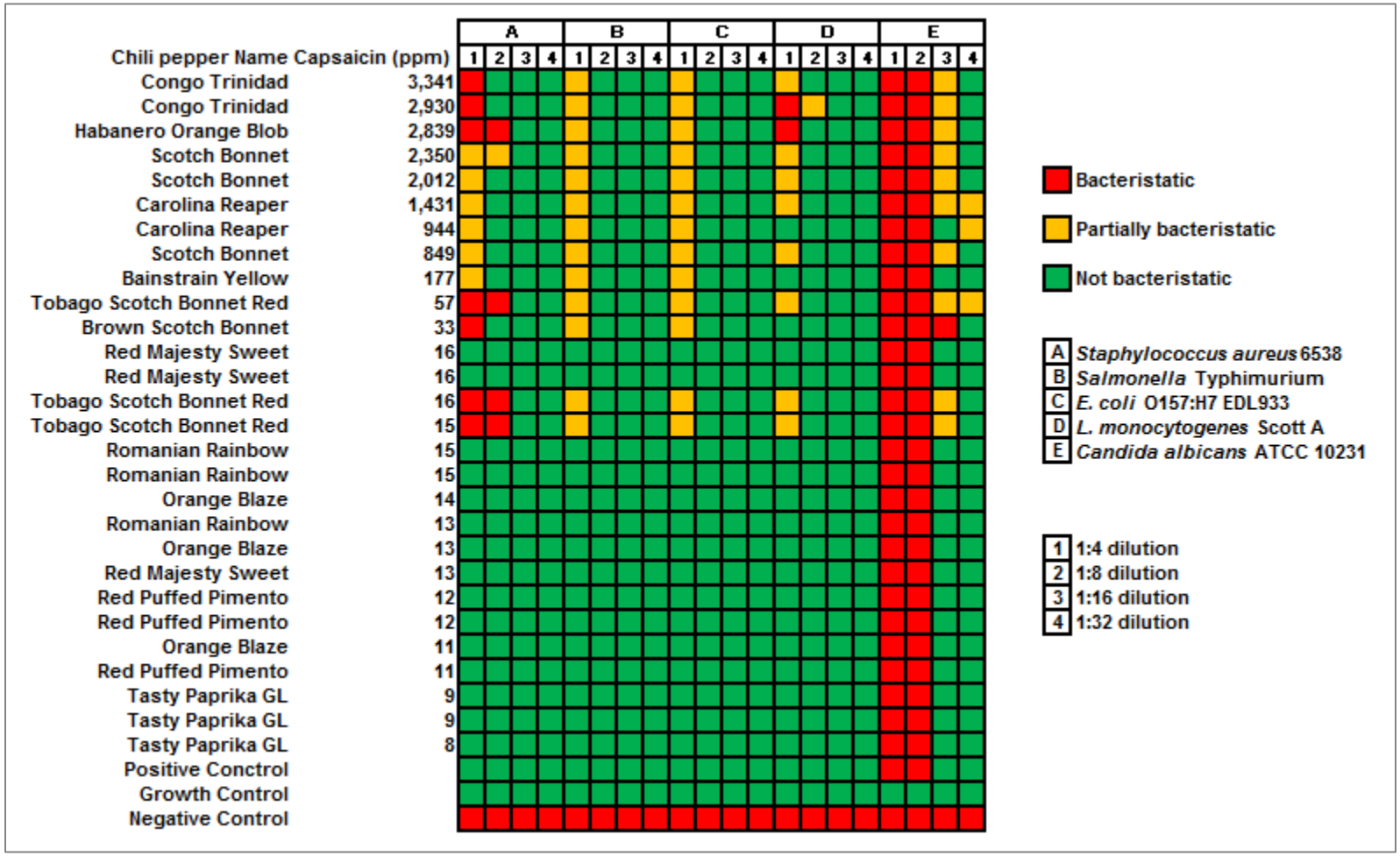

Figure 11: Effects of crude chili pepper extracts on microbial growth

4.5 Discussion

The methanol extracts inhibited Gram positive and partially inhibited Gram negative bacteria in a dose dependent manner. Gram negative bacterial cell walls have an outer impermeable membrane which makes them intrinsically resistant to certain antimicrobials either because they lack the target of the antimicrobial or because the antimicrobial cannot get into the cytoplasm (44, 56). Suffredini *et al*. (57) analyzed 12 extracts obtained from nine plants belonging to six different genera of Clusiaceae against Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) bacteria using the microdilution broth assay. Suffredini *et al.* reported that Gram negative bacteria are hardly susceptible to the plant extracts in doses less than 2×10^5 μg/mL. Our sample with the highest capsaicin content only had 63,308 μg/ml capsaicin.

Mills-Robertson *et al.* (19) investigated the MICs of the ethanol extract of *Cryptolepis sanguinolenta* and its partitioned fractions by the microplate dilution method. All the extract and fractions showed MIC values ranging from 500 μg/ml to 32,000 μg/ml but not all were bactericidal. Navarro et al.[37] investigating 12 methanolic plant extracts normally used in traditional medicine in Mexico to cure infectious diseases, examined the potential antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. They reported significant antimicrobial effects, with MICs ranging between 600 and 40,000 μg/ml of crude extract against the

microbes. We determined that the MIC values for 50% natural capsaicin ranged from 50 ppm for *Candida albicans* to greater than 200 ppm for *E. coli* O157:H7. This data is consistent with the viability test and growth inhibition test results. It would be necessary to determine the percentage purity of the capsaicin in the chili pepper samples if reasonable comparisons are to be made between the chili pepper sample data these MIC values.

Findings by Nascimento *et al*. (43), show a remarkable antimicrobial activity of capsaicin isolated from extracts of *Capsicum* frutescence (Pimenta Malagueta), against Gram negative bacteria ranging from 0.06 to 10 µg/ml. To put these values in context, extracts with MIC ≤ 100 µg/ml and isolated compounds with MIC ≤ 10 µg/ml are considered very interesting (58). Nascimento *et al*. (43) determined that the minimum inhibitory concentration of capsaicin for the growth of *E.coli*, *Staphylococcus aureus* and *Candida albicans* were 5, 1.2 and 25 µg/ml respectively. It took higher concentrations of capsaicin to inhibit *Enterococcus faecalis*, *Bacillus subtilis* and *Pseudomonas aeruginosa* as compared to *Staphylococcus aureus*, *Klebsiella pneumoniae* and *E. coli*. This could be attributed to the fact that some bacteria use capsaicin as a nutrient for growth (59). In our study, a similar phenomenon was observed. In samples that did not contain enough capsaicin to inhibit growth, the bacterial cells seemed to grow better than the positive and growth controls. If the cells exposed to the sub-lethal levels of capsaicin were able to utilize these potential "carbon sources", it would make sense that they grew better than the growth control.

According to a previous study by Molina-Torres *et al*. (15), capsaicin concentrations of up to 200 or 300 µg/ml only retarded the growth of *E. coli.* This is consistent with our findings, but contradicts the study by Nascimento *et al*. (43), who determined that the MIC for *E.coli* was 5 µg/ml. Our study determined that the MIC for 99.1% pure synthetic capsaicin against *E.coli* O157:H7 was 100 µg/ml. However, when 50% natural capsaicin was used, the highest concentration tested (200 µg/ml) only retarded the growth of *E. coli* O157:H7. The study by Nascimento *et al*. (43), further contradicts a study by Dorantes *et al.* (6), who did not find any antimicrobial activity for capsaicin against *B. subtilis, Staphylococcus aureus* and *Candida albicans* .

Cichewitz *et al.* (4) chose to use the disc diffusion method for their study. The *Capsicum* tissue extracts tested were blended without a solvent and filtered through a cheese cloth. The extracts exhibited a variety of effects with the test organisms, including complete inhibition, partial inhibition, stimulation, and partial inhibition with an outer zone of stimulation. Cooking the *Capsicum* extract altered its activity by increasing or decreasing the diameter of the zone of inhibition or altering the inhibitory effect from complete to partial inhibition, partial to complete inhibition, inhibition to no inhibition, or no inhibition to inhibition. Because capsaicin is thermal stable, this change in inhibitory effects brings into question what compound in the extracts was actually responsible for the effects Cichewitz *et al* (4) observed. There was no mention of the results of the test against *Salmonella* Typhimurium, *E. coli* and *Staphylococcus aureus*. Tests against *Candida albicans* demonstrated slightly enhanced growth in most cases.

Cichewitz *et al.* (4) tested 10 mg/ml of capsaicin (60 % and 98% purity) against *B. cereus, B. subtilis, Candida albicans , Clostridium sporogenes, E. coli, Salmonella* Typhimurium, *Staphylococcus aureus*, and *Streptococcus pyogenes* and noted no inhibitory effects. This study by Cichewitz *et al.* (4), is particularly interesting because 10 g/ml is a very high concentration. All the other research findings cited in this study involved the testing on capsaicin in µg/ml. It is possible that the disc diffusion method used by Cichewitz *et al.* (4), was not very effective. Our team tried this method and decided that the growth inhibition assay offered better sensitivity.

Careaga *et al*. (13), determined that the minimum lethal concentration of *Capsicum annuum* type bell pepper against *Salmonella* Typhimurium ATCC 14028 was 1.5 ml/100g beef. Because bell peppers generally do not contain capsaicin, and the researchers did not specify which type of bell pepper they used, it is difficult to compare our data to the results obtained by Careaga *et al*. (13). However, the results from Careaga *et al*. (13) are similar to those obtained by Dorantes *et al* (6), who tested habanero (5880 µg/ml capsaicin), serrano (630 µg/ml capsaicin) and pimiento morron peppers (no capsaicin) against *L. monocytogenes* Scott A, *Staphylococcus aureus* FRI-S6, and *Salmonella* Typhimurium ATCC 13311. According to Dorantes *et al* (6), the pimiento pepper, which has no capsaicin, showed the highest levels of activity against all three pathogens. *L. monocytogenes* was the most sensitive to the chili extracts and *Salmonella* Typhimurium was the least sensitive.

Dorantes *et al* (6) suggest, after determining the HPLC profile of the three peppers, that coumaric acid and cinnamic acid were most likely responsible for the antimicrobial activity of the pimiento pepper (the data was not shown). Dorantes *et al.* (6) further suggested that the habanero pepper, which contained the highest levels of capsaicin (but no coumaric or cinnamic acids), was the least effective antimicrobial. The serrano pepper which contained capsaicin (630 µg/ml), cinnamic (470 µg/ml) and coumaric (900 µg/ml) acids showed activity, but not as much as the pimiento pepper. In our study, the MIC for coumaric acid was also investigated. As previously mentioned, concentrations greater than 200 µg/ml (highest tested), are needed to inhibit the growth of all five microbes tested. Additionally, we did not note any inhibitory effects from chili pepper samples that were low in capsaicin, except in the case of Trinidad Scotch Bonnet Red.

4.6 Conclusion

There are a variety of methods for testing the antimicrobial activities of chili peppers. These methods strongly affect the observed levels of inhibition. Various reasons may contribute in the differences between results, including inconsistency between analyzed plant materials (10). Dorantes *et al*. concluded that the capsaicinoids composition of chili pepper extracts are different and this may influence their antimicrobial effects (6). From our data, it is reasonable to conclude that the chili pepper

samples that contained small amounts of capsaicin had no effect on microbial viability or growth.

A realistic next step would be to determine whether or not there are synergistic effects between capsaicin and coumaric acid. One way to do this would be to simply prepare samples with synthetic versions of both compounds and test these against pathogens of interest as mentioned previously in this study. The other option would be to determine the HPLC profile of the chili peppers that showed antimicrobial activity, as shown in Figures 10 and 11. This would help us understand whether or not these peppers contain coumaric acid in addition to capsaicin.

Nascimento *et al.*(43) identified a new chili pepper flavonoid called chrysoeriol and suggested that it had the best antimicrobial activity against the pathogens tested, as previously mentioned. However, the MIC value for *Candida albicans* was not detected. They suggest further tests to identify other possible biological and industrial applications of this compound.

The other factor worth considering in the tolerance the average population has for capsaicin, if it is to be considered for food preservation. Al Othman *et al.* (35), reported the mean consumption of *Capsicum* spices to be 2.5 g/person/day in India, 5 g/person/day in Thailand and 20 g/person (corresponding to one chili pepper) per day in Mexico. Assuming a content of capsaicinoids in these spices of about 1%, the daily intake of capsaicinoids in these countries has been estimated to 25,000 – 200,000 µg/person/day, which corresponds in the case of a person with 50 kg body weight to 500

– 4,000 µg/kg bw/day. The maximum daily intake of capsaicin in the U.S. and Europe from mild chilies and paprika was estimated to be roughly 25 µg/kg bw/day, which is equivalent to 1500 µg/person/day. According to a recent estimation, the mean and maximum intakes of capsaicin from industrially prepared food products containing the recommended general limit of 5 µg/g, would be 770 and 2640 µg/day, respectively. These number are much lower than the concentrations needed to kill pathogens such as *E. col*i and Salmonella. Therefore, the application of hurdle technology (the use of multiple methods to ensure food safety), would still be necessary.

This study offered a few highlights in the continued pursuit of natural antimicrobial properties of chili peppers. To the best of our knowledge, this is the first study to grow all the tested chili peppers under the same conditions and investigate them for antimicrobial properties. Additionally, this is the first study where tests were carried out on chili peppers hotter than a habanero. We used 29 chili peppers from the widest variety tested to date. We were also the first to test for both bactericidal and bacteriostatic effects of chili peppers.

5 Summary

Culturability is not always a perfect indicator of viability. Comparing Figure 10 and 11 clearly indicates that there are instances when cells are viable, but not able to grow due to the stress exerted on them by their environment. In this case, some of the chili pepper samples had no observable effect on viability, but inhibited growth. This information is useful when considering antimicrobials for food safety. In short, if the bacterial cells can overcome the stress from their environment, they resume normal growth and this makes food unsafe.

Also, the use of sub-lethal levels of antimicrobials is not wise. As noted in this study, and by Nascimento *et al*. (43), some bacteria can metabolize capsaicin as a sole carbon source. Therefore, using concentrations of capsaicin that do not kill the microbes, might make things worse, by providing an additional carbon source for their growth.

The major take away from this study is the diversity of data collected by different researchers. As previously stated, the type of chili peppers tested, the climate, and testing methods all affect the data obtained. In addition, testing crude extracts still leaves the question of what compound in the chili peppers are antimicrobial. Capsaicin, coumaric and cinnamic acids have all been implicated as antimicrobials by various studies, as we showed in the literature review. Nascimento *et al*. (43) identified chrysoeriol as another possible candidate.

It seems clear that a few things need to be standardized, before the various studies can be reasonably compared. The first is growth conditions. As long as chili peppers are

grown under entirely different environmental conditions, comparing data between studies remains a challenge. Secondly, test methods for determining antimicrobial activities should also be standardized. For example, the capsaicin assay is standard and all studies can easily compare these data.

An obvious concern is whether or not these tested pathogens could potential develop resistance to capsaicinoids as they do other antimicrobials. Understanding the mechanism by which capsaicin affects microbial cells would be invaluable in determining the potential use of chili peppers as food preservatives.

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