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Antimicrobial activity of aqueous Yerba Mate extracts

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To the Graduate Council:

I am submitting herewith a dissertation written by Kellie Parks Burris entitled "Antimicrobial activity of aqueous Yerba Mate extracts." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

Federico M. Harte, Major Professor

We have read this dissertation and recommend its acceptance:

C. Neal Stewart Jr., Philip M. Davidson, Svetlana Zivanovic

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Antimicrobial activity of aqueous Yerba Mate extracts

A Dissertation presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Kellie Parks Burris
May 2011

Dedication

This dissertation is dedicated to my husband and best friend

Jason Burris

whose love, patience and aggravation allowed for its completion.

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I would like to offer my sincere thanks and appreciation to Dr. Federico Harte for taking on the role of my major professor in Food Science and Technology. It was through his favorite beverage that allowed this research to get started. I would like to sincerely thank my co-advisor and mentor, Dr. C. Neal Stewart, Jr., for providing me funding to pursue my degree in Food Science and Technology. It is through his support that my research was started and completed. I would also like to thank my committee members, Dr. P. M. Davidson, who was helpful and encouraging with anything in projects relating to microbiology and to Dr. Svetlana Zivanovic, who was helpful with anything in projects relating to food chemistry. I would like to thank my committee as a whole for providing suggestions on my research and writing.

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Abstract

Ilex paraguariensis, is used in the preparation of a widely popular tea beverage (Yerba Mate) mainly produced and consumed in the countries of Uruguay, Paraguay, Argentina and Brazil. Dialyzed aqueous extracts were screened for antimicrobial activity against *Escherichia coli* O157:H7, *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *S. pseudintermedius*, and *Pseudomonas syringae* pv. tomato.

Using a concentrated extract, *S. aureus* was found to be the more sensitive to extracts than *E. coli* O157:H7. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of extract tested that did not allow bacterial growth (inhibition) above the original inoculums of approximately 5.0-6.0 log CFU/ml after 24 hr. The minimum bactericidal concentration (MBC) was defined as the lowest concentration tested where bacterial death (inactivation) was observed after 24 hr. MBCs were determined to be ca. 0.150- 0.800 mg protein equivalent/ml and 0.025-0.050 mg protein equivalent/ml against *E. coli* O157:H7 and *S. aureus* respectively.

Using a lyophilized extract, MICs were determined to be 5 mg/ml for two strains of *E. coli* O157:H7 and MBCs 5 mg/ml for *E. coli* O157:H7 strain ATCC 43894 and 10 mg/ml for *E. coli* O157:H7 strain 'Cider' in microbiological media. An approximately >4.5 log reduction was observed for *E. coli* O157:H7 treated with 40 mg/ml extract in modified apple juice, which approximate to the requirements of the United States Code of Federal Regulations (21 CFR part 120).

We demonstrated antimicrobial effectiveness of aqueous extracts after 24 hr at 1 and 2 mg/ml against all strains of methicillin-resistant *S. pseudintermedius* and of *S. aureus* tested respectively. An approximately >5 log reduction was observed in all strains at all concentrations after 24 hr. Methicllin-resistant *Staphylococcus aureus* (MRSA) strains appeared more susceptible to the extract than methicillin-resistant *Staphylococcus pseudintermedius* (MSRP) strains.

It was concluded that aqueous extracts of Yerba Mate demonstrated broad activity against foodborne, human, animal and plant pathogenic bacteria, including strains demonstrating resistance to certain antibiotics.

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Chapter 1: Introduction

Pathogens are a major concern for a variety of industries—food, human and animal health, and plant. Foodborne illnesses are a continuous threat to public health and Scallan et al. (2011) estimate that the 31 major foodborne pathogens account for nearly 9.4 million people becoming sick, more than 55,961 hospitalizations, and 1,351 deaths from foodborne illness each year in the United States with an estimated economic cost of $152 billion annually (Scharff, 2010). Further, losses in major crops due to fungal and bacterial pathogens ranges from 7-22%, with losses reported for potatoes (22%), wheat (16%), rice (16%), barley (15%), maize (10%), cotton (9%), sugar beet (8%), and soybeans (7-16%) (Oerke and Dehne, 2004). There are many methods of inhibiting or inactivating microorganisms, however, interest in the use of natural plant-derived products versus chemicals as antimicrobials is increasing significantly, including their use in the protection of foods and crops. Further, various bacteria have developed resistance to certain antibiotics, and thus, other forms of bactericidal agents are required.

For thousands of years, plants have been used as a rich source of bioactive compounds. Plants are continually attacked by a multitude of pathogens and have adapted natural defense mechanisms which protect them from developing disease. Plants are able to resist disease through the production of reactive oxygen species, certain antimicrobial compounds and have the ability to alert surrounding tissues and plants through signaling molecules. Of the 252 drugs considered essential by the World Health Organization, 11% were derived from flowering plants (Rates, 2001). With the increased demand for more organic and naturally produced foods, researchers are looking to obtain novel, natural bioactive compounds from botanicals for use in a variety of commercial applications requiring elimination and/or inhibition of pathogenic and spoilage bacteria, including but not limited to the protection of plants, medicines, cosmetics, and

animal/human feeds/foods. Further, these antimicrobials should be safe, effective, novel and bio-based agents.

Yerba Mate is processed from the leaves and small stems of *I. paraguariensis* and is consumed socially as a non-alcoholic beverage, and as coffee, primarily for its caffeine content. *I. paraguariensis*, a native South American holly shrub from the Aquifoliaceae family, is primarily produced and consumed in the countries of Argentina, Brazil, Paraguay, and Uruguay (Grigioni et al., 2004). Yerba Mate is typically consumed from a small cup or "mate" by regularly pouring small amounts of hot water over a serving, approximately 50 g of packed tea. The beverage is drunk through a metal straw or "bombilla", which has small holes that prevents ingesting the large particles or leaves (Kubo et al., 1993; Heck and De Mejia, 2007; Heck et al., 2008).

Yerba Mate has been researched mainly for its antioxidant, anticancer, anti-inflammation, and anti-atherosclerosis qualities (Gugliucci and Stahl, 1995; Carini et al., 1998; Anesini et al., 2006; Heck and de Mejia, 2007). However, little research has been conducted on determining the compounds responsible for antimicrobial activity from Yerba Mate (*Ilex paraguariensis*) in comparison to other plant-based beverages such as green tea or coffee. Within the last two decades, a surge in research examining this botanical for its use in human health has occurred, with a more recent focus on its antimicrobial activity (Hongpattarakere, 2000; Burris et al., 2011a; Burris et al., 2011b).

Many of the major compounds found in Yerba Mate extracts are known (Kubo et al. 1993; Hongpattarakere, 2000; Heck and de Mejia, 2007); however, conflicting information exists on

which compounds might contribute to its antimicrobial activity and whether these compounds may have additive or synergistic effects when used in combination. The main compounds found in Yerba Mate that may be contributing to antimicrobial activity include caffeic acid, caffeine, caffeoyl derivatives, caffeoylshikimic acid, chlorogenic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin and theobromine (Heck and de Mejia, 2007; Marques and Farah, 2009).

Some individual compounds identified from Yerba Mate in their pure forms have demonstrated antimicrobial activity. For example, caffeic and chlorogenic acids in their pure form have demonstrated activity against Gram-negative bacteria (Herald and Davidson, 1983; Puupponen-Pimia et al., 2001). However, Kubo et al. (1993) found that the three main compounds found in Yerba Mate, caffeine, ursolic acid and chlorogenic acid, did not demonstrate antimicrobial activity against Gram-negative or Gram-positive bacteria, and Rauha et al. (2000) found caffeic acid did not demonstrate inhibitory activity against the Gram-positive bacteria, *S. aureus*, *S. epidermidis*, or *Bacillus subtilis*, providing conflicting results to Herald and Davidson (1983) and Puupponen-Pimia et al. (2001). Herald and Davidson (1983) demonstrated a reduction in viable *S. aureus* at pH 5.0 by p-coumeric acid. Caffeine was not shown to inhibit *Streptococcus mutans* (Daglia et al., 2002), indicating caffeine was not contributing to activity observed by Kubo et al. (1993). Several of the flavonols, kaempferol, quercetin and rutin found in Yerba Mate have also been examined for their antimicrobial activity (Rauha et al., 2000; Panizzi et al., 2002). Kaempferol did not to inhibit *S. epidermidis* (Rauha et al., 2000) or *E. coli* (Puupponen-Pimia et al., 2001), however, it demonstrated antimicrobial activity against the Gram-positive bacteria, *S. aureus* (Rauha et al., 2000). Similarly, quercetin exhibited strong inhibition against *S.*

aureus, but unlike kaempferol, provided strong to moderate activity against *S. epidermidis* and *B. subtilis* respectively (Rauha et al., 2000). Results from Panizzi et al. (2002) were contradictory to these, where neither kaempferol nor quercetin demonstrated antimicrobial activity against *S. aureus* or *E. coli*. Rutin did not demonstrate any activity against *S. aureus*, *S. epidermidis* or *B. subtilis* (Rauha et al., 2000). Caffeoylquinic acid derivatives have been shown to contribute to antimicrobial activity in other crude plant extracts (Chakraborty and Mitra, 2008) and have been found in Yerba Mate extract (Filip et al., 2000, 2010). Research remains on the identification of those compounds responsible for antimicrobial activity and it may be likely a combination of compounds found in Yerba Mate extracts is contributing to the antimicrobial activity against Gram-negative and Gram-positive bacteria as indicated by the ineffectiveness of activity of some individual identified compounds. While we may not know the absolute identification of individual compounds contributing to activity, the crude extracts and several isolated compounds derived from Yerba Mate have been shown active against a broad spectrum of Gram-positive and Gram-negative bacteria (Kubo et al., 1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et al., 2008; Burris et al., 2011a). This evidence points to the potential use of crude extracts and fractionatated extracts as novel antimicrobials for use in foods and crops.

The goal of this research was to determine if aqueous extracts of Yerba Mate derived from *I. paraguariensis* demonstrated broad activity against foodborne, human, animal and plant pathogenic bacteria, including strains demonstrating resistance to certain antibiotics. Extracts with this type of activity can be utilized in a variety of industries requiring elimination and inhibition of pathogenic bacteria, including but not limited to the protection of plants, medicines, cosmetics, and animal/human feeds/foods.

This dissertation is organized into seven chapters. This introduction provides a short background to the importance of novel antimicrobials, specifically addressing the benefits of Yerba Mate. Chapter 2 is a literature review on the potential of Yerba Mate as a source of antimicrobial activity and is in manuscript format. Chapters 3-6 encompass the breadth of the research and are also provided in manuscript format. Specifically, chapters are separated as follows: initial studies of Yerba Mate aqueous extracts against foodborne pathogens (Chapter 3), use of Yerba Mate aqueous extracts in apple juice (Chapter 4), use of Yerba Mate aqueous extracts against methicillin-resistant staphylococci (Chapter 5), and use of Yerba Mate aqueous extracts against plant pathogens (Chapter 6). Finally, conclusions are discussed.

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Chapter 2: Composition and bioactive properties of Yerba Mate (*Ilex paraguariensis*): A review.

Adapted from:

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Composition and bioactive properties of Yerba Mate (*Ilex paraguariensis*): A review. Chilean Journal of Agricultural Research. Submitted.

I wrote the manuscript.

2.1 Abstract

Yerba Mate is a popular tea beverage produced and consumed in the South American countries of Argentina, Brazil, Chile, Paraguay, and Uruguay and is processed from the leaves and stems of *Ilex paraguariensis*, a perennial shrub from the Aquifoliaceae (holly) family. Production occurs in six stages: harvesting older leaves and small stems, roasting by direct fire, drying under hot air, milling to specified size, aging to acquire optimal sensory attributes, and final packaging. While grown and consumed for centuries in South America, its popularity is increasing in the United Sates because of the demand by consumers for healthier, more natural foods, is filling a niche for a different type of tea beverage and for its potential health benefits—antimicrobial, antioxidant, antiobesity, anti-diabetic, digestive improvement, stimulant, and cardiovascular properties. Cultivation, production and processing may cause a variation in bioactive compounds biosynthesis and degradation. Recent research has been expanded to its potential use as an antimicrobial, protecting crops and foods against foodborne, human and plant pathogens. Promising results for the use of this botanical in human and animal health has prompted this review. This review focuses on the known chemical composition of Yerba Mate, the effect cultivation, production and processing may have on composition, along with a specific discussion of those compounds found in Yerba Mate that have antimicrobial properties.

Key words: antioxidant, antimicrobials, natural products, Yerba mate, tea.

RESUMEN

Yerba Mate es una infusión popular producida y consumida en Argentina, Brasil, Chile, Paraguay y Uruguay. Se procesa a partir de hojas y tallos de *Ilex paraguariensis*, un arbusto perenne de la familia Aquifoliaceae. El procesamiento ocurre en seis etapas: recolección de hojas maduras y tallos pequeños, tostado por fuego directo, secado por aire caliente, molienda, envejecimiento (dependiendo de los atributos sensoriales requeridos), y embalaje final. Si bien Yerba Mate se ha cultivado y consumido por siglos en América del Sur, su popularidad en los Estados Unidos ha aumentando debido a la demanda por bebidas saludables y alimentos más naturales y por las potenciales potencialmente beneficiosas de la Yerba Mate (antixidante, antimicrobiano, acción contra la obesidad y diabetes, digestivo, estimulante). La Yerba Mate también se ha investigado como agente de prevención y causa de algunos tipos de cáncer, causando controversia entre investigadores. Investigaciones recientes han ampliado el espectro de uso de la Yerba mate como agente antimicrobiano, protección de cultivos y la acción contra patógenos transmitidos por alimentos. Resultados prometedores para el uso de este botánico en la salud humana y animal ha llevado a esta revisión. Esta revisión se centra en la composición de la Yerba Mate, y el efecto que el cultivo y el procesamiento puede tener sobre sus propiedades.

Palabras clave: antioxidantes, antimicrobianos, natural, Yerba mate, té.

2.2 Introduction

Little research has been conducted on the bioactive and chemical composition of Yerba Mate (*I. paraguariensis*) in comparison to other plant-based beverages such as green tea or coffee. However, within the last two decades, there has been an increased interest in this botanical for its use in human health. *I. paraguariensis*, a native South American holly shrub from the Aquifoliaceae family, is mainly produced and consumed in the countries of Argentina, Brazil, Chile, Paraguay, and Uruguay (Grigioni et al., 2004). Yerba Mate, processed from the leaves and small stems of *I. paraguariensis* (Fig. 2-1), is a non-alcoholic beverage consumed socially primarily in these countries and like coffee, primarily for its caffeine content. Typical consumption of Yerba Mate is taken from a small cup or "mate". Small amounts of hot water are regularly poured over a serving, ca. 50 g of packed tea. The beverage is then drunk by using a metal straw or "bombilla", which has small holes that prevent the leaves from being consumed (Kubo et al., 1993; Heck and De Mejia, 2007; Heck et al., 2008).

Argentina was the first significant exporter of Yerba Mate and is second behind Uruguay for highest per capita consumption (Heck and de Mejia, 2007). In 2004, the total value of Mate production in the world was estimated at $1 billion (Heck and de Mejia, 2007). Yerba Mate is commercially produced from plants grown and harvested from plantations and natural forests (Heck et al., 2008). Once harvested, commercial Yerba Mate undergoes a series of processing steps before the final product is packaged—harvesting, roasting, drying, milling and aging. Specific steps vary depending on the geographic region where the tea will be consumed (Heck and De Mejia, 2007; Heck et al., 2008). Several recent studies have examined the phytochemical content of Yerba Mate under different growth and processing conditions (Schmalko and

Alzamora, 2001; Esmelindro et al., 2002; Giulian et al., 2009; Isolabella et al., 2010). Growing and processing conditions both have been shown to have an effect on the chemical composition of the *I. paraguariensis* (Heck et al., 2008), thus influencing the pharmacological properties.

The composition of Yerba Mate has been partially characterized and it includes a variety of polyphenols, xanthines, caffeoyl derivatives, saponins, and minerals that may be responsible for pharmacological activity (Alikaridis,1987; Gosmann and Schenkel, 1989; Clifford and Ramirezmartinez, 1990; Carini et al., 1998; Filip et al., 2001; Bastos et al., 2006; Bastos et al., 2007; Bravo et al., 2007; Cardozo et al., 2007; Heck and de Mejia, 2007; Marques and Farah, 2009) (Table 1). Studies have suggested that consumption of Yerba Mate leaves may have antioxidant (Gugliucci and Stahl, 1995; Carini et al., 1998; Filip et al., 2000; Anesini et al., 2006; Bastos et al., 2006; Bastos et al., 2007; Pagliosa et al., 2010), antiobesity (Andersen and Fogh, 2001), antidiabetic (Lunceford and Gugliucci, 2005), diuretic (Gorgen et al., 2005), chemopreventative, antifungal (Filip et al., 2010), and stimulant (Filip et al., 1998; Athaydel et al., 2000) properties. It may also aid in digestion (Gorzalczany et al., 2001). Yerba Mate has been recognized for a variety of pharmacological activities, but limited research has been conducted on its antimicrobial properties (Kubo et al., 1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et al., 2008; Burris et al., 2011).

The use of Yerba Mate as an antimicrobial in foods and for crop protection is a relatively new concept (Racanicci et al., 2009; Burris et al., 2011) and has not been fully studied and reviewed. Crude extracts, i.e. tea, and a variety of isolated compounds derived from Yerba Mate have been shown active against a broad spectrum of Gram-positive and Gram-negative bacteria (Kubo et al.,

1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et al., 2008; Burris et al., 2011). This points to possible use of crude extracts and isolated compounds as novel antimicrobials in foods.

The objective of this paper is to review the composition of Yerba Mate, the effect cultivation, production and processing has on its composition, and focus on those compounds that have bioactive properties.

2.3 Cultivation and processing

Yerba Mate can be cultivated and processed in a variety of ways. Typically, Yerba Mate is grown in two different environments—plantations or natural forests. Plantations are the more popular growth environment because of ease of harvest and a more consistent production quality and quality (Heck et al., 2008). Yerba Mate processing occurs in six steps: harvesting, roasting, drying, milling aging and blending/packing (Isolabella et al., 2010). The leaves and small stems are harvested mechanically, divided into 100 kg sacks, and transported to a processing facility. Roasting, which inactivates enzymes and preserves sensory qualities, occurs by direct contact with fire at temperatures between 250 °C and 550 °C for 2 to 4 min (Isolabella et al., 2010). Drying takes place by exposure to hot air until moisture content of 3% is attained. The drying process typically takes 12 to 14 hr (Isolabella et al., 2010). Heck et al. (2008) examined the effects of growing and drying conditions on the phenolic composition of Yerba Mate, and found plantation grown Yerba Mate had higher levels of phenolic acids compared to forest grown-Mate (Heck et al., 2008). Cultivation and processing can have a significant effect on the production and concentration of phytochemicals.

2.4 Primary chemical composition of Yerba Mate

2.4.1 Phenolic compounds

Structurally, polyphenols are comprised of a benzene ring that is bound with one or more hydroxyl groups. Polyphenols are derived from Mate plants and are considered its major bioactive compounds. The level of polyphenolics in Yerba Mate extracts are greater than those of green tea and similar to levels found in red wine (Gugliucci et al., 2009; Gugliucci and Bastos, 2009). The polyphenols found in Yerba Mate include caffeic acid, caffeine, caffeoyl derivatives, caffeoylshikimic acid, chlorogenic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin, and theobromine (Carini et al., 1998; Chandra and de Mejia Gonzalez, 2004; Atoui et al., 2005; Bastos et al., 2007, Bravo et al., 2007) with caffeoyl derivatives accounting for approximately 10% of the dry weight (Filip et al., 2001) (Table 2-1). A number of growing and processing factors can affect the amount of polyphenols extracted from Yerba Mate (Heck et al., 2008; Isolabella et al., 2010). Additionally, the method of consumption can have an influence on extracted polyphenolics (Meinhart et al., 2010). A total infusion preparation with cold water, termed 'terere', demonstrated the extraction of almost all phenolics (Meinhart et al., 2010). It was found that green leaves contained significantly lower concentrations of active compounds, caffeoyl derivative, methylxanthines and flavonoids, as compared to those leaves that had undergone processing, drying and aging (Isolabella et al., 2010). Yerba Mate extracts are highly rich in chlorogenic acids, and unlike green tea, contain no catechins (Chandra and de Mejia Gonzalez, 2004). According to (Dall'Orto et al., 2005), on average, approximately 92 mg equivalent chlorogenic acid was extracted from each gram of Yerba Mate leaves. (Jaiswal et al., 2010) detected and characterized 42 chlorogenic acids isomers based from Yerba Mate using LC-MS—eight caffeoylquinic acids, five dicaffeoylquinic acids, six feruloylquinic acids, two

diferuloyl quinic acids, five p-coumaroylquinic acids, four caffeoyl-p-coumaroylquinic acids, seven caffeoyl-feruloylquinic acids, three caffeoyl-sinapoylquinic acids, one tricaffeoylquinic acid, and one dicaffeoyl-feruloylquinic acid.

The polyphenolic content of Yerba Mate has been shown to be strongly related to its overall antioxidant capacity (Chandra and de Mejia Gonzalez, 2004) similar to green tea. Polyphenols are reducing agents and have been reported to provide body tissues protection from oxidative stress that causes aging, cancer, cardiovascular disease and inflammation (Ames et al., 1993; Scalbert et al., 2005).

2.4.2 Saponins

Saponins are glycosidic compounds that are generally water-soluble and foam upon shaking (Bastos et al., 2007). The primary saponins identified from Yerba Mate are matesaponin 1 through 5 (Gosmann and Schenkel, 1989; Gosmann et al., 1995; Kraemer et al., 1996). Matesaponin 1 was first discovered by Gosmman et al. (1989) with a chemical structure of ursolic acid-3-0-[β-D-glucopyranosyl-(1→3)-α-L-arabino-pyranosyl]-(28→1)-β-D-glucopyranosyl ester. Gosmann et al. (1995) then discovered and structurally determined three more saponins, matesaponin 2 through 4. Yerba Mate leaves have a relatively high saponin content, 5 to 10% of the total dry weight. Puangpraphant et al. (2011) quantified and purified saponins from dried Mate leaves and obtained 10 to 15 mg/g dry weight total saponins, mainly matesaponins 1 and 2 (Puangpraphant et al., 2011). The method of consumption of Yerba Mate influences the amount of xanthines extraction. Meinhart et al. (2010) determined that the highest quanities of xanthines were extracted from partial infusions with hot water.

Saponins have been reported to provide a hypocholesteremic effect by inhibiting the passive diffusion of colic acid through the formation of micelles preventing absorption, anticancer, antiparasitic (Taketa et al., 2004a,b), and anti-inflammatory properties (Puangpraphant et al., 2009, 2011).

2.4.3 Xanthines

Xanthines are a class of purine alkaloids found in many different plants. There are three xanthines found in Yerba Mate, caffeine, theobromine, and theophyline, and give Yerba Mate its characteristic bitter flavor and stimulant effects (Filip et al., 1998; Athayde et al., 2000; Gorgen et al., 2005). Of these, caffeine is present in the highest concentrations at 1 to 2% of total dry weight, followed by theobromine at 0.3 to 0.9% of total dry weight (Ito et al., 1997) (Table 2-1). The consumption of caffeine found in a cup of Yerba Mate (78 mg) is similar to that of a cup of coffee (85 mg); however, the typically method of Yerba Mate consumption involving repeatedly pouring additional hot water over in the 'mate' can yield intakes greater than 260 mg of caffeine, attributed to percent stem or woody content and extraction rate (Mazzafera, 1997). Processing by three-stage drying was shown to significantly decrease caffeine content by 30% (Schmalko and Alzamora, 2001). However, Bastos et al. (2006) found that dried leaves had significantly higher amounts of caffeine than fresh leaves most likely due to the increased soluble solids content lending to a more efficient extaction.

2.5 Bioactive properties and health implications

2.5.1 Antimicrobial and oral health

Relatively limited research has been conducted on isolation and identification of compounds possessing antimicrobial activity derived from Yerba Mate (Kubo et al., 1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et al., 2008; Filip et al., 2010). Kubo et al. (2010) identified caffeoyl derivatives, methylxanthines, and rutin from Yerba Mate aqueous extracts with antifungal activity. N-hexane extracts of Yerba Mate have been shown to be effective antimicrobial agents against the oral bacterium, *Streptococcus mutans* (Kubo et al., 1993). The 10 main compounds identified as potential antimicrobial components were linalool, α-ionone, β-ionone, α-terpineol, octanoic acid, geraniol, 1-octanol, nerolidol, geranylactetone and eugenol (Kubo et al., 1993). These compounds have been shown to be active against a broad spectrum of Gram-positive and Gram-negative bacteria, with effective levels between 12.5 and 1600 μg ml^{-1} (Taniguchi et al., 1978; Kubo et al., 1991, 1993; Sari et al., 2007). The Gram-positive bacteria, *Bacillus subtilis, Brevibacterium ammoniagenes, Propionibacterium acnes, Staphylococcus aureus,* and *Streptococcus mutans,* and five fungi, *Saccharomyces cerevisiae, Candida utilis, Pityrosporum ovale, Penicillium chrysogenum,* and *Trichophyton mentagrophytes,* were inhibited by at least one of the ten identified compounds tested (Kubo et al., 1993). None of the extracts tested were effective against the Gram-negative bacteria, *Pseudomonas aeruginosa* or *Enterobacter aerogenes* and were found to be only weakly active against *Escherichia coli* (Kubo et al., 1993). Burris et al. (2011) determined that aqueous extracts from Yerba Mate demonstrated antimicrobial activity against *S. aureus* and *E. coli* O157:H7, indicating inhibition and inactivation of both Gram-positive and Gram-negative bacteria. This finding suggests that an

additional compound is present in the aqueous extract that provides activity in addition to the 10 identified by Kubo et al. (1993).

While many of the major compounds found in Yerba Mate extracts are known (Kubo et al. 1993; Hongpattarakere, 2000; Heck and de Mejia, 2007), contradictory information is available on which compounds might contribute to antimicrobial activity and whether they may have additive or synergistic effects in combination. Polyphenols identified in Yerba Mate include caffeic acid, caffeine, caffeoyl derivatives, caffeoylshikimic acid, chlorogenic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin and theobromine (Heck and de Mejia, 2007; Marques and Farah, 2009) all of which may contribute to the antimicrobial activity against foodborne pathogens. Caffeic and chlorogenic acids in their pure form have demonstrated activity against Gram-negative bacteria (Herald and Davidson, 1983; Puupponen-Pimia et al., 2001). However, Kubo et al. (1993) found that the three main compounds found in Yerba Mate, caffeine, ursolic acid and chlorogenic acid, did not demonstrate antimicrobial activity against Gram-negative or Gram-positive bacteria, including *E. coli* and *S. aureus.* Further, Rauha et al. (2000) found caffeic acid did not demonstrate inhibitory activity against the Gram-positive bacteria, *S. aureus*, *S. epidermidis*, or *Bacillus subtilis.* However, Herald and Davidson (1983) demonstrated a reduction in viable *S. aureus* at pH 5.0 by p-coumeric acid. No inhibition against *Streptococcus mutans* has been observed with caffeine (Daglia et al., 2002), indicating caffeine was not contributing to activity observed by Kubo et al. (1993). Several of the flavonols found in Yerba Mate have also been examined for their antimicrobial activity—kaempferol, quercetin, and rutin (Rauha et al., 2000; Panizzi et al., 2002). Kaempferol did not to inhibit *S. epidermidis* (Rauha et al., 2000) or *E. coli* (Puupponen-Pimia et al., 2001); however, it demonstrated antimicrobial

activity against *S. aureus* (Rauha et al., 2000). Similarly, quercetin exhibited strong inhibition against *S. aureus*, but unlike kaempferol, provided strong to moderate activity against *S. epidermidis* and *B. subtilis* respectively (Rauha et al., 2000). Results from Panizzi et al. (2002) were contradictory to these, where neither kaempferol nor quercetin demonstrated antimicrobial activity against *S. aureus* or *E. coli*. Rutin did not demonstrate any activity against *S. aureus*, *S. epidermidis* or *B. subtilis* (Rauha et al., 2000).

Caffeoylquinic acid derivatives have been shown to contribute to antimicrobial activity in other crude plant extracts (Chakraborty and Mitra, 2008) and have been found in Yerba Mate extract (Filip et al., 2000, 2010). It is likely a combination of compounds found in Yerba Mate extracts is contributing to the antimicrobial activity against Gram-negative and Gram-positive bacteria as evidenced by the ineffectiveness of activity of some individual compounds.

2.5.2 Antioxidant, Anti-obesity, Anti-inflammation

The pharmacological properties—antioxidant, anti-obesity and anti-inflammation—of Yerba Mate extracts and compounds have been previously reviewed (Bastos et al., 2007; Heck and de Mejia, 2007; Bracesco et al., 2010). Oxygen radicals are involved in many human disease including cancer, inflammation, liver and cardiovascular disease (Ames et al., 1993; Halliwell, 1994). Yerba Mate extracts have been previously shown to provide antioxidant activity and inhibition of low-density-lipoproteins oxidation (Gugliucci and Stahl, 1995; Filip et al., 2000; Chandra and de Mejia Gonzalez, 2004) *in vitro* (Gugliucci and Stahl, 1995; Filip et al., 2000) and *in vivo* (Gugliucci, 1996; Schinella et al., 2000; Lanzetti et al., 2008). Similarly, Martins et al. (2009) determined that mice fed Yerba Mate had lower ThioBarbituric Acid Reactive

Substances in the liver, suggesting that treatment with Yerba Mate extract protected unsaturated fatty acids from oxidation and may especially protect the liver (Martins et al., 2009).

According to Anderson and Fogh (2001), in overweight patients, Yerba Mate extract significantly delayed gastric emptying, decreased the perceived time to fullness and ultimately induced a significant weight loss after 45 days (Arcari et al., 2009) demonstrated that treatment with Yerba Mate extract has potent anti-obesity effects in adipose tissue *in vivo* by controlling the expression of several genes related to obesity processes, such as inflammatory markers.

Inflammation is a factor in many human diseases—cancer, cardiovascular disease, obesity and diabetes. Lanzetti et al. (2008) determined that Yerba Mate reduced acute lung inflammation in mice exposed to cigarette smoke. Recently, Puangpraphant and de Mejia (2009) investigated the potential anti-inflammatory effect of Yerba Mate extracts as well as some of its compound and their interactions. Quercetin was determined the most potent inhibitor of pro-inflammatory responses at a concentration 10 times lower than that of other tested compounds (Puangpraphant and de Mejia, 2009).

2.6 Conclusions

Plants have been an abundant source of bioactive compounds for thousands of years. However, within the last decade, the want and need for more natural, bioactive compounds has grown. Research on extracts and isolated compounds from Yerba Mate to benefit human health has provided a number of pharmacological applications: antioxidant, antimicrobial, anti-inflammatory, antiobesity, and anticancer. With the potential use of Yerba Mate extracts as

antimicrobials in foods, sensory qualities must be addressed. One common negative factor associated with the use of plant extracts as antimicrobial food preservatives is their affect on sensory (flavor, odor) properties of the food. The flavor of Yerba Mate infusions has been described in various terms, such as bitter, acid, astringent, hay, green, humid, toasted, and paper (Cruz et al., 2003). However, use of Yerba Mate (dried and aqueous extracts) had no effect on the taste or smell of precooked chicken meat balls (Racanicci et al., 2009).

While the need for more research on the isolation and identification of bioactive compounds exists, evidence seems to demonstrate that Yerba Mate is a botanical with a variety of compounds that can be applied for use in human health. Research confirms the influence cultivation and processing have on the chemical composition of Yerba Mate and demonstrates their importance in the production of bioactive compounds. Further research can be explored to optimize growth and processing technologies to enhance bioactive compounds for use in foods, crops, cosmetics, neutraceuticals, and supplements to support human health.

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Appendix

Table and Figure

Table 2-1. Main bioactive compounds found in Yerba Mate and their potential health benefits.

Chemical compound	**ca. % dry wt. composition**	**Potential health benefits**	**Reference**
Caffeoyl derivatives	10.000		(Filip et al., 2001)
Chlorogenic acid	2.800	Antioxidant, **antimicrobial,** antidiabetic, analgesic	(Filip et al., 2001)
Caffeic acid	0.023	Antioxidant	(Filip et al., 2001; Heck and de Mejia, 2007; Filip et al., 2000)
3, 4-DCQ	0.855	Anticancer, antioxidant	(Filip et al. 2001; Arbiser et al., 2005)
3, 5-DCQ	3.040	Anticancer, antioxidant	(Filip et al., 2001; Arbiser et al., 2005)
4, 5-DCQ	2.890		(Filip et al., 2001)
Saponins	5 to 10	Anticancer, anti-inflammation, antiparasitic	(Puangpraphant et al., 2011; Taketa et al., 2004b)
Xanthines			
Caffeine	1 to 2%	Anticarcinogenic, antiobesity, antioxidant, diuretic, stimulant, vasodialator	(Ito et al. 1997; Heck and de Mejia, 2007)
Theobromine	0.3 to 0.9%	Stimulant, diuretic	(Ito et al. 1997; Heck and de Mejia, 2007)
Theophylline	0 to trace	Stimulant, vasodialator	(Ito et al. 1997; Heck and de Mejia, 2007)
Rutin	0.060	Antioxidant, lipoxygenase-inhibitor, anticancer, anti-tumor, anti-ulcer	(Arbiser et al., 2005; Heck and de Mejia, 2007)
Quercetin	0.0031	Anticancer, anti-inflammation, **antimicrobial**	(Puangpraphant and de Mejia, 2009; Arbiser et al., 2005; Rauha et al., 2000)
Kaempferol	0.0012	Anti-inflammation, **antimicrobial**	(Puangpraphant and de Mejia, 2009; Rauha et al., 2000)

Figure 2-1. Yerba Mate (*Ilex paraguariensis*) plant grown in the greenhouse in Knoxville, TN. Approximately 24 mo old and 6 ft tall.

Chapter 3: Antimicrobial activity of Yerba Mate (*Ilex paraguariensis*) aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus*

Adapted from:

Kellie P. Burris, Philip M. Davidson, Charles N. Stewart, Jr. and Federico M. Harte. Antimicrobial activity of Yerba Mate (*Ilex paraguariensis*) aqueous extracts against *Escherichia coli* O157:H7 and *Staphylococcus aureus*. Journal Food Science. Accepted.

I designed, executed and analyzed the experiments and wrote the manuscript.

3.1 Abstract

There are numerous methods for inhibiting or inactivating microorganisms. However, the use of natural plant products is becoming increasingly important to a variety of industries. *Ilex paraguariensis* is used in the preparation of a widely popular tea beverage in South America (Yerba Mate) in the countries of Uruguay, Paraguay, Argentina and Brazil. In this study, extracts of four brands of commercial tea, derived from the holly plant species, *I. paraguariensis*, were evaluated for their ability to inhibit or inactivate bacterial foodborne pathogens. The ultimate goal was to evaluate potential use of the extracts in commercial applications. Dialyzed aqueous extracts were screened for antimicrobial activity against *Escherichia coli* O157:H7 and *Staphylococcus aureus*. *S. aureus* was found to be the more sensitive to extracts than *Escherichia coli* O157:H7. Minimum bactericidal concentrations MBCs were determined to be approximately 150- 800 μg protein equivalent ml^{-1} and 25-50 μg protein equivalent ml^{-1} against *E. coli* O157:H7 and *S. aureus* respectively. A Uruguayan brand had reduced activity against *E. coli* O157:H7 compared to the Argentinean brands tested. It was concluded that Yerba Mate could be used as a potential antimicrobial in foods and beverages against Gram-positive and Gram-negative pathogenic bacteria.

Keywords: Aqueous tea extract, Yerba Mate, *Ilex paraguariensis*, antimicrobial activity, foodborne pathogens

Practical application

Soluble extracts from Yerba Mate are natural antimicrobials that can be incorporated into food products to achieve longer shelf life.

3.2 Introduction

Yerba Mate is a tea beverage largely consumed in the South American countries of Brazil, Argentina, Paraguay and Uruguay and is increasing in popularity in the United States as a food ingredient or in dietary supplements (Heck et al., 2008). Yerba Mate is prepared from a blend of dried leaves and/or stems from *I. paraguariensis*, a native South American holly shrub from the Aquifoliaceae family (Small and Catling, 2001; Grigioni et al., 2004; Heck et al., 2008). Typically, approximately 50 g of dried tea is packed into a cup or "mate" and small amounts of hot water are regularly poured and then removed by suction using a metal straw or "bombilla". The bottom end of the bombilla has small holes that prevent the leaves from being removed while the infusion is drunk (Kubo et al., 1993; Heck and de Mejia, 2007; Heck et al., 2008). Commercial Yerba Mate undergoes a series of processing steps before the final product is packaged—blanching, drying, milling and aging. Specific steps vary depending on the geographic region where the tea will be consumed (Heck and de Mejia, 2007; Heck et al., 2008). The composition of Yerba Mate has been partially characterized and it includes a variety of polyphenols, xanthines, caffeoyl derivatives, saponins, and minerals (Alikaridis, 1987; Gosmann et al., 1989; Clifford and Ramirezmartinez, 1990; Carini et al., 1998; Filip et al., 2000; Bastos et al., 2006, 2007a,b; Bravo et al., 2007; Cardozo et al., 2007; Heck and de Mejia, 2007; Marques and Farah, 2009). Both growing and processing conditions have an effect on the chemical composition of the *I. paraguariensis* (Heck et al., 2008). For example, plantation grown Yerba Mate plants were found to have higher concentrations of polyphenols than those grown in the forest (Heck et al., 2008). Studies have suggested that consumption of Yerba Mate leaves may have antioxidant (Gugliucci and Stahl, 1995; Carini et al., 1998; Filip et al., 2000; Anesini et al., 2006; Bastos et al., 2006, 2007a,b; Pagliosa et al., 2010), antiobesity (Andersen and Fogh, 2001;

Bastos et al., 2007b), antidiabetic (Bastos et al., 2007b), and diuretic capacity (Bastos et al., 2007b; Gorgen et al., 2005), chemopreventative, antifungal (Filip et al., 2010), and stimulant (Bastos et al., 2007b) properties. It may also aid in digestion (Gorzalczany et al., 2001; Bastos et al., 2007b). While Yerba Mate has been recognized for a variety of pharmacological activities, limited research has been conducted on its antimicrobial properties (Kubo et al., 1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et al., 2008).

E. coli O157:H7 and *S. aureus* are two important foodborne pathogens that cause many cases and illnesses annually. The type of illness caused by foodborne *E. coli* O157:H7 or *S. aureus* are infections or intoxications, respectively. There are numerous physical and chemical methods for inhibiting or inactivating microorganisms in foods; however, there is an increased interest in the use of natural antimicrobials for preservation of foods. One common negative factor associated with the use of plant extracts as antimicrobial food preservatives is their affect on sensory (flavor, odor) properties of the food. The flavor of Yerba Mate infusions has been described in various terms, such as bitter, acid, astringent, hay, green, humid, toasted, and paper (Cruz et al., 2003). However, use of Yerba Mate (dried and aqueous extracts) had no effect on the taste or smell of precooked chicken meat balls (Racanicci et al., 2009).

The objective of this study was to determine if Yerba Mate had antimicrobial activity against two foodborne bacterial pathogens, *E. coli* O157:H7 and *S. aureus*. Four brands of commercial Yerba Mate tea, derived from *I. paraguariensis*, were tested for their ability to inhibit or inactivate the pathogens.

3.3 Materials and Methods

3.3.1 Culture preparation

E. coli O157:H7 ATCC 43889 and strain 'Cider' were stock cultures obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville and *S. aureus* ATCC 27708 and strain SA113 were obtained from the Center Environmental Biotechnology at the University of Tennessee, Knoxville (courtesy of Dr. Steven Ripp). All cultures were grown in tryptic soy broth (TSB; Difco, Sparks, MD) and stored at -20°C. Working cultures were obtained by inoculating 50 ml TSB with 100 µl stock cultures and incubating for 24 hr at 35-37°C. After incubation, the cultures were diluted to approximately 4.0-5.0 log CFU (colony forming units) ml^{-1} and tested for antimicrobial activity.

3.3.2 Aqueous extraction

Dried leaves of three brands of Yerba Mate (Nobleza Gaucha, Rosamonte, Taragui) were purchased from a local international supermarket and one brand of Yerba Mate (La Mulata Suave) was acquired directly in Uruguay. Dried Yerba Mate tissue was flash frozen in liquid nitrogen and finely ground with a mortar and pestle. Extracts were obtained by using a HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer protein extraction protocol (Markham et al., 2006). Finely-ground tissue was resuspended in extraction buffer (20 mM HEPES pH 8, 0.5 mM EDTA (ethylenediaminetetraacetic acid), 10% glycerol, 1 mM phenylmethylsulfonylfluoride, and 1 mM benzamidine) at a ratio of 1 g tissue to 3.6 ml buffer and was allowed to stand for 2 hr at 4 °C with occasional mixing to maximize extraction. The aqueous extracts were then dialyzed against 20 mM HEPES for 36 h using a 3500 MWCO

SnakeSkin® Pleated Dialysis Tubing (Pierce Biotechnology, Rockford, IL) to remove secondary metabolites and other low molecular weight compounds used in the extraction buffer. The resulting extracts were centrifuged at 5000 x g for 30 min to remove sediment and concentrated to approximately 2 mg equivalent protein per ml buffer using Vivaspin Spin Concentrators MWCO 5000 (Sartorius Corporation, Edgewood, NY). This process was repeated until a sufficient quantity of extract had been collected to allow for the antimicrobial assays. Concentrated extracts were aliquotted and stored at -80°C. Bradford assays (Bradford Plus; Pierce) were performed to determine total protein content (Bradford 1976) which was used to adjust the concentration for inactivation assays. Protein equivalents of approximately 2 mg ml^{-1} diluted in 20 mM HEPES were added to time kill assays at 40% of the total volume.

3.3.3 Time kill assays

Dialyzed aqueous extracts (0-800 μg ml^{-1} protein equivalent) diluted in 20 mM HEPES were mixed with bacteria harvested at late logarithmic phase and diluted to approximately 4.0-5.0 log CFU ml^{-1}. A total volume of 25 ml was used consisting of 12.5 ml of TSB, 10 mL of filtered extract or 20 mM hepes buffer (positive control), and 2.5 ml of inoculum. Bacteria and extracts were incubated in TSB at 35-37°C and at regular intervals (0, 3, 6, 12 and 24 hr) a bacterial suspension sample (1 or 0.1 ml) was collected, serially diluted in 0.1% peptone, plated in duplicate (1 or 0.1 ml) using tryptic soy agar (TSA; TSB, Becton, Dickinson and Company, Sparks, MD and Agar, Fisher Scientific, USA), incubated for 24 hr at 35-37°C, and then CFU enumerated. All experiments were duplicated and average values were reported. MIC and MBC were determined for each brand and strain combination. MIC was determined as the lowest concentration tested that did not demonstrate bacterial growth (inhibition) above the original

inoculums approximately 4.0-5.0 log CFU ml^{-1} after 24 hr. The MBC was determined as the lowest concentration tested where bacterial death (inactivation) was observed after 24 hr.

3.3.4 Statistical analysis

Data were analyzed as a completely randomized design with two replicates by analysis of variance (ANOVA) using the general linear model (SAS 9.2, SAS Institute, Cary, NC). Least significant differences (LSD) were used to compare treatment mean values when significant differences (at the 0.10 probability level) were found. Error bars represent 95% confidence intervals using LSD.

For examining strain and treatment (brand/concentration) differences, data were analyzed as a completely randomized design with sampling and nested treatment design when comparing means for *E. coli* O157:H7 and as a completely randomized design with sampling and factorial treatment design when comparing means for *S. auerus* by ANOVA. ANOVA was performed using mixed models (SAS, 9.2), and least square means were computed and separated using LSD ($P<0.05$).

3.4 Results and Discussion

Extractions from the four commercial brands of tea yielded relative large quantities of protein (approximately 100 mg from approximately 100 g tissue). Protein equivalents were used to determine concentrations applied to antimicrobial assays.

The dialyzed aqueous extracts of Yerba Mate were evaluated for growth inhibition and inactivation activity against *E. coli* O157:H7 and *S. aureus*. Overall, aqueous dialyzed extracts from four brands of commercially available Yerba Mate, derived from *I. paraguariensis*, were effective against both Gram-positive and Gram-negative foodborne pathogens. By using multiple brands of commercial available Yerba Mate tea, we concluded that the activity of the tea extracts was due to compounds found naturally in the processed leaves and stems of Yerba Mate as opposed to contaminants during commercial processing. Antimicrobial activity was observed against both Gram-negative and Gram-positive using tea quantities less than or comparable to typical consumption (50 g), with activities occurring at 1-20 g of dried tea equivalent depending upon bacterium tested.

Figs. 3-1 through 3-4 indicate the inhibitory effects of Yerba Mate tea extracts on the growth of *E. coli* O157:H7 and *S. aureus*. Among the four brands of Yerba Mate tested, aqueous extracts were effective as antimicrobials against *S. aureus* strains ATCC 27708 (Fig. 3-1) and SA113 (Fig. 3-2) and *E. coli* O157:H7 strains ATCC 43894 (Fig. 3-3) and Cider (Fig. 3-4). MICs and MBC required for inhibition and inactivation of *S. aureus* strains ATCC 27708 and SA113 and *E. coli* O157:H7 strains ATCC 43894 and 'Cider' are compiled in Table 3-1. MICs were determined to be 12.5 μg protein equivalent ml^{-1} for all brands against *S. aureus* ATCC 27708 (Fig. 3-1) and <50 μg protein equivalent ml^{-1} for La Mulata, Rosamonte and Taragui and 25 μg protein equivalent ml^{-1} for Nobleza Gaucha against *S. aureus* SA113 (Fig. 3-2). MBCs were slightly higher and determined to be 25 μg protein equivalent ml^{-1} for La Mulata, Nobleza Gaucha and Rosamonte and 50 μg protein equivalent ml^{-1} for Taragui against *S. aureus* ATCC

27708 (Fig. 3-1) and ≤50 μg protein equivalent ml^{-1} for all brands against *S. aureus* SA113 (Fig. 3-2).

Higher concentrations of each brand were required to inhibit and inactivate *E. coli* O157:H7 as compared to *S. aureus*. MICs were determined to be 200 μg protein equivalent ml^{-1} for La Mulata and 150 μg protein equivalent ml^{-1} for Nobleza Gaucha, Rosamonte and Taragui against *E. coli* O157:H7 ATCC 43894 and 800 μg protein equivalent ml^{-1} for all brands tested against *E. coli* O157:H7 'Cider' (Fig 3-4.). MBCs were determined to be 150 μg protein equivalent ml^{-1} and 800 μg protein equivalent ml^{-1} for Nobleza Gaucha, Rosamonte and Taragui against *E. coli* O157:H7 ATCC 43894 and 'Cider' respectively (Figs. 3-4). The Uruguayan brand, La Mulata, was only tested against *E. coli* O157:H7 strain ATCC 43894 and did not demonstrate bactericidal activity at any of the concentrations tested (Fig. 3-3A). Since La Mulata was not effective against the more sensitive strain of *E. coli* O157:H7 ATCC 43894 (Fig. 3-3A), it was unlikely to be effective against the more resistant strain. La Mulata (Fig. 3-3A) demonstrated lower activity against *E. coli* O157:H7 than any of the Argentinean brands tested (Fig. 3-3B-D).

Table 3-2 and 3-3 provide an overview of strain and brand/concentration effects for *S. aureus* and *E. coli* O157:H7 respectively. For *S. aureus*, main effects of the two treatment factors (strain and brand) were important ($P<0.001$), but the interaction between strain and brand was not ($P=0.8946$). For *E. coli* O157:H7, the main effect of strain was not important ($P=0.7121$). However, the strain nested within brand was significant ($P<0.05$).

An aqueous extraction protocol was used to obtain extracts with antimicrobial activity against both Gram-positive and Gram-negative pathogenic bacteria. Crude extracts and many individual components in green and black teas have demonstrated broad activity against Gram-positive and Gram-negative bacteria (Kubo et al., 1992; Yam et al., 1997; Taguri et al., 2004; Yoda et al., 2004; Si et al., 2006;). However, relatively limited research has been performed on isolation and identification of compounds possessing antimicrobial activity derived from Yerba Mate (Kubo et al., 1993; Hongpattarakere 2000; Sari et al., 2007; Tsai et al., 2008). N-hexane distillate extracts of Yerba Mate have been shown to be effective antimicrobial agents against the oral bacterium, *Streptococcus mutans* (Kubo et al., 1993). The 10 main compounds that were identified in the distillate using GC-MS were linalool, α-ionone, β-ionone, α-terpineol, octanoic acid, geraniol, 1-octanol, nerolidol, geranylactetone and eugenol (Kubo et al., 1993). The MICs of these individual components were determined between 12.5 and 1600 $\mu g\ ml^{-1}$ for a broad spectrum of Gram-positive and Gram-negative bacteria (Taniguchi et al., 1978; Kubo et al., 1991, 1993; Sari et al., 2007). Gram-positive bacteria, *Bacillus subtilis, Brevibacterium ammoniagenes, Propionibacterium acnes, S. aureus,* and *Streptococcus mutans,* and five fungi, *Saccharomyces cereuisiae, Candida utilis, Pityrosporum ovale, Penicillium chrysogenum,* and *Trichophyton mentagrophytes,* were inhibited by at least one of the 10 volatile compounds tested (Kubo et al., 1993). None of the distillate compounds tested were effective against the Gram-negative bacteria, *Pseudomonas aeruginosa* or *Enterobacter aerogenes* and were only weakly active against *E. coli* (Kubo et al., 1993). However, compounds present in our aqueous extracts demonstrating antimicrobial activity may be different than those reported by Kubo et al., (1993). We determined inhibition and inactivation of both Gram-positive and Gram-negative bacteria

whereas Kubo et al., (1993) and Sari et al., (2007) only demonstrated weak or no activity respectively against *E. coli*.

In agreement with the current study, Hongpattarkere (2000), using disk diffusion assays, determined the activity of aqueous and organic extracts of Yerba Mate against Gram-positive and Gram-negative bacteria and observed strong inhibition of *S. aureus* using aqueous extracts. While we observed bactericidal effects against *E. coli* O157:H7, Hongpattarakere (2000) observed little inhibition of the bacterium using water-based extraction.

While many of the compounds found in Yerba Mate extracts are known (Kubo et al., 1993; Hongpattarakere 2000; Heck and de Mejia 2007), limited information is available on which compounds might contribute to antimicrobial activity and whether they may have additive or synergistic effects in combination. The three characteristic compounds found in Yerba Mate, caffeine, ursolic acid and chlorogenic acid, did not demonstrate antimicrobial activity against Gram-negative or Gram-positive bacteria, including *E. coli* and *S. aureus*, at the highest concentration (400 $\mu g\ ml^{-1}$) tested (Kubo et al., 1993). Polyphenols identified in Yerba Mate include caffeic acid, caffeine, caffeoyl derivatives, caffeoylshikimic acid, chlorogenic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin and theobromine (Heck and de Mejia 2007; Marques and Farah 2009) and may contribute to the antimicrobial activity against *E. coli* O157:H7 and *S. aureus*. Caffeic and chlorogenic acids in their pure form have demonstrated activity against Gram-negative bacteria (Herald and Davidson, 1983; Puupponen-Pimia et al., 2001). Rauha et al. (2000) found caffeic acid did not demonstrate inhibitory activity against the Gram-positive bacteria, *S. aureus*, *S. epidermidis*, or *Bacillus subtilis.* However, Herald and

Davidson (1983) demonstrated a reduction in viable *S. aureus* at pH 5.0. No inhibition against *Streptococcus mutans* has been observed with caffeine (Daglia et al., 2002). Several of the flavonols found in Yerba Mate have been examined for their antimicrobial activity—kaempferol, quercetin, and rutin (Rauha et al., 2000; Panizzi et al., 2002). Kaempferol was found not to inhibit *S. epidermidis* (Rauha et al., 2000) or *E. coli* (Puupponen-Pimia et al., 2001); however, it demonstrated clear antimicrobial activity against *S. aureus* (Rauha et al., 2000). Similarly, quercetin exhibited strong inhibition against *S. aureus*, but unlike kaempferol, demonstrated strong to moderate activity against *S. epidermidis* and *B. subtilis* respectively (Rauha et al., 2000). This is somewhat contradictory to results from Panizzi et al. (2002) which demonstrated no antimicrobial activity of kaempferol or quercetin against *S. aureus* or *E. coli*. Rutin did not demonstrate any activity against *Staph aureus*, *S. epidermidis* or *B. subtilis* (Rauha et al., 2000). Caffeoylquinic acid derivatives have been shown to contribute to antimicrobial activity in other crude plant extracts (Chakraborty and Mitra 2008). Perhaps, one or more compounds are contributing to the antimicrobial activity against Gram-negative and Gram-positive bacteria in this study.

The role of drinking Yerba Mate may have with the prevalence of certain cancers is a controversial topic. Research suggests that temperature of consumption and introduction of carcinogenic contaminants during processing may contribute to cancer risks (Loria et al., 2009). While many studies have been conducted examining Yerba Mate as an anti-cancer agent, a recent meta-analysis performed by Dassanayake et al. (2010) demonstrated the potential association of drinking Mate with increased incidences in oral and oral-pharyngeal cancers in hospital-based case-control studies. However, to eliminate confounding factors, Loria et al.,

(2009) suggest that future studies should include population-based rather than hospital-based case-control studies on Mate consumption and cancer risk, and data gathered should include specifics on tobacco, alcohol, hot beverages, fresh fruit and vegetables use as well as a better method of measuring accurate volumes and temperatures of Yerba Mate consumed. While this extract has potential for use in foods, more definitive research, including human and animal studies, needs to be conducted before a conclusion can be reached on its potential cancer risk.

3.5 Conclusions

One of the primary objectives of the food and beverage industry is to protect consumers from the harmful effects of foodborne pathogens. Dialyzed aqueous extracts of Yerba Mate demonstrated bactericidal activity against Gram-positive and Gram-negative human and foodborne pathogens. Our results demonstrated that relatively low concentrations of Yerba Mate aqueous extracts provide antimicrobial activity against Gram-positive and Gram-negative bacteria. Furthermore, the compounds responsible for antimicrobial activity appear to be very stable since these results were found using starting materials that had undergone commercial preparation of blanching, drying, milling and aging. The use of natural plant products versus purified or synthesized chemicals as antimicrobials is becoming increasingly popular. Exploring the potential for use of antimicrobials derived from natural sources or bio-based compounds is important in finding alternatives or adjuncts to regulatory-approved traditional antimicrobials.

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Appendix

Tables and figures

Table 3-1. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations (μg protein equivalents/ml) required for inhibition and inactivation of *S. aureus* strains ATCC 27708 and SA113 and *E. coli* O157:H7 strains ATCC 43894 and 'Cider'.

	S. aureus				***E. coli* O157:H7**			
	ATCC 27708		**SA113**		**ATCC 43894**		**'Cider'**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Brand	**μg protein equivalents/ml**							
La Mulata	6.25	25	25	50	200	No kill	ND	ND
Nobleza Gaucha	6.25	25	12.5	25	150	150	200	800
Rosamonte	6.25	25	12.5	25	150	150	200	800
Taragui	6.25	25	25	50	150	150	200	800

ND: not determined

Table 3-2. Mean estimates of the interaction between *S. aureus* strains ATCC 27708 and SA113 and treatment (brand and concentration) (La Mulata, Nobleza Gaucha, Rosamonte and Taragui at 50, 25, 12.5, and 6.25 μg/ml). LSD was used to compare mean estimates of the interaction of strain and treatment (P<0.05). Columns and rows with different letters are statistically significant (P<0.05).

Brand	**Concentration (μg protein equivalent/ml)**	**ATCC 27708**	**SA113**
La Mulata	50	1.0722 K	2.6702 GHIJK
	25	2.5227 GHIJK	4.5418 CDEFG
	12.5	4.7157 BCDEFG	6.2066 ABCDE
	6.25	7.1599 AB	6.6095 ABCD
Nobleza Gaucha	50	1.1748 K	2.5008 GHIJK
	25	1.3476 JK	3.2347 GHIJK
	12.5	4.1189 EFGHI	5.8596 ABCDEF
	6.25	6.1293 ABCDEF	6.5532 ABCDE
Rosamonte	50	1.0170 K	2.4071 GHIJK
	25	1.7240 IJK	4.3861 DEFG
	12.5	3.6789 FGHIJ	5.8996 ABCDEF
	6.25	6.1790 ABCDE	6.8211 ABCD
Taragui	50	1.0393 K	1.9031 HIJK
	25	2.5312 GHIJK	4.6405 CDEFG
	12.5	4.4403 CDEFG	6.3604 ABCDE
	6.25	6.8912 ABC	6.7833 ABCD
Control	0	7.5937 A	7.1636 AB

Table 3-3. Mean estimates of the interaction between *E. coli* O157:H7 strains ATCC 43894 and 'Cider' and treatment (brand and concentration) (La Mulata, Nobleza Gaucha, Rosamonte and Taragui at 800, 400, 200, 100 or 200, 150, 100, and 50 μg/ml). Data were analyzed as a completely randomized design with sampling and nested treatment design. LSD was used to compare mean estimates of the interaction of strain and treatment (P<0.05). Columns and rows with different letters are statistically significant (P<0.05).

Brand	**Concentration (μg protein equivalent/ml)**	**ATCC 43894**	**'Cider'**
La Mulata	200	5.8553 ABCDEF	ND
	150	6.7660 AB	ND
	100	7.3745 AB	ND
	50	7.8547 AB	ND
Nobleza Gaucha	800	ND	2.3524 H
	400	ND	5.3555 BCDEFG
	200	2.9535 FGH	5.9879 ABCDE
	150	3.3638 DEFGH	ND
	100	6.7115 ABC	6.9949 AB
	50	7.8694 AB	ND
Rosamonte	800	ND	3.7890 CDEFGH
	400	ND	6.0028 ABCDE
	200	3.0776 EFGH	6.2632 ABCD
	150	3.3668 DEFGH	ND
	100	6.4111 ABC	6.9539 AB
	50	7.8087 AB	ND
Taragui	800	ND	2.8150 GH
	400	ND	5.4996 ABCDEFG
	200	3.0828 EFGH	6.8588 AB
	150	3.4413 DEFGH	ND
	100	7.0912 AB	7.2454 AB
	50	7.8342 AB	ND
Control	0	8.3476 A	7.8733 AB

ND: Not determined

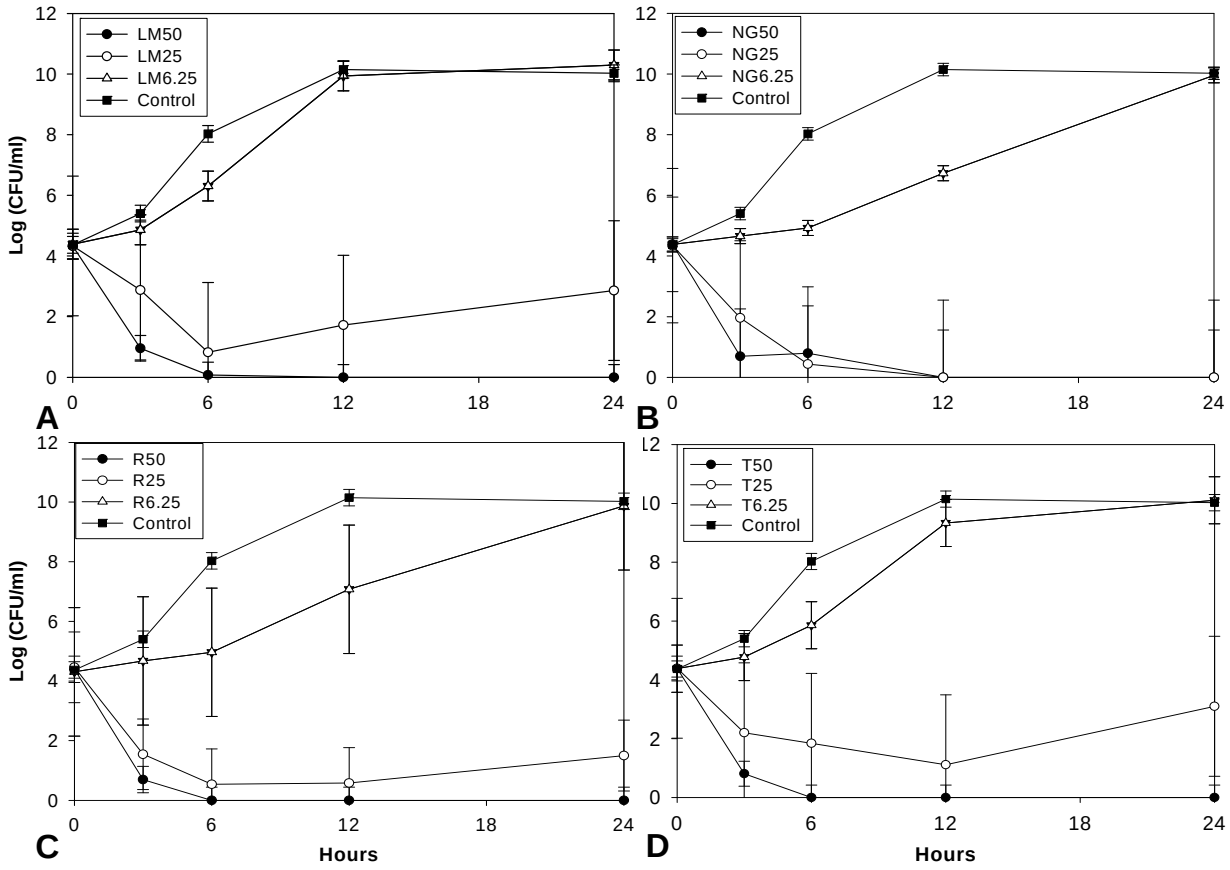

Figure 3-1. Antimicrobial activity of Yerba Mate tea, (A) LM: La Mulata, (B) NG: Nobleza Gaucha, (C) R: Rosamonte and (D) T: Taragui, aqueous extracts at 50, 25, and 6.25 μg ml^{-1} against *Staphylococcus aureus* 27708. Error bars represent 95% confidence intervals using least significant differences (P<0.10).

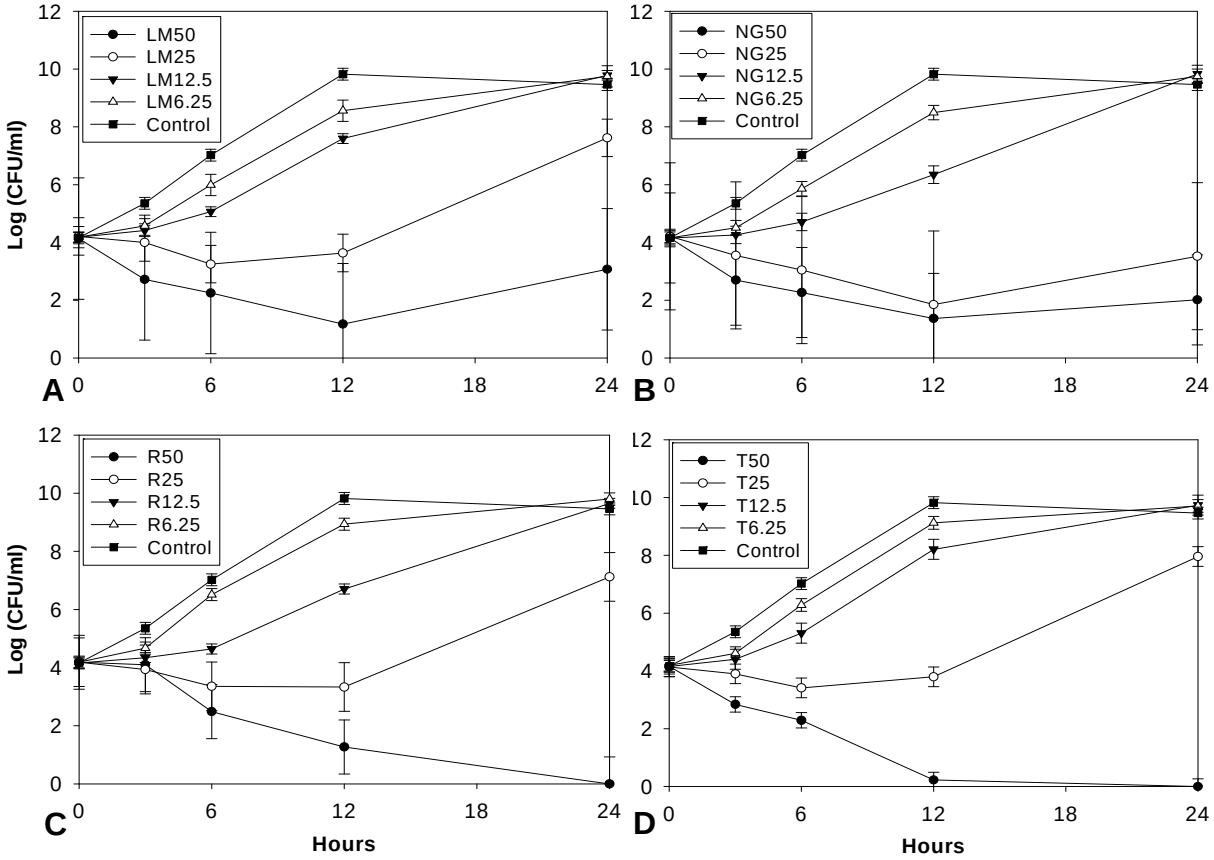

Figure 3-2. Antimicrobial activity of Yerba Mate tea, (A) LM: La Mulata, (B) NG: Nobleza Gaucha, (C) R: Rosamonte and (D) T: Taragui, aqueous extracts at 50, 25, 12.5 and 6.25 μg ml^{-1} against *Staphylococcus aureus* SA113. Error bars represent 95% confidence intervals using least significant differences ($P<0.10$).

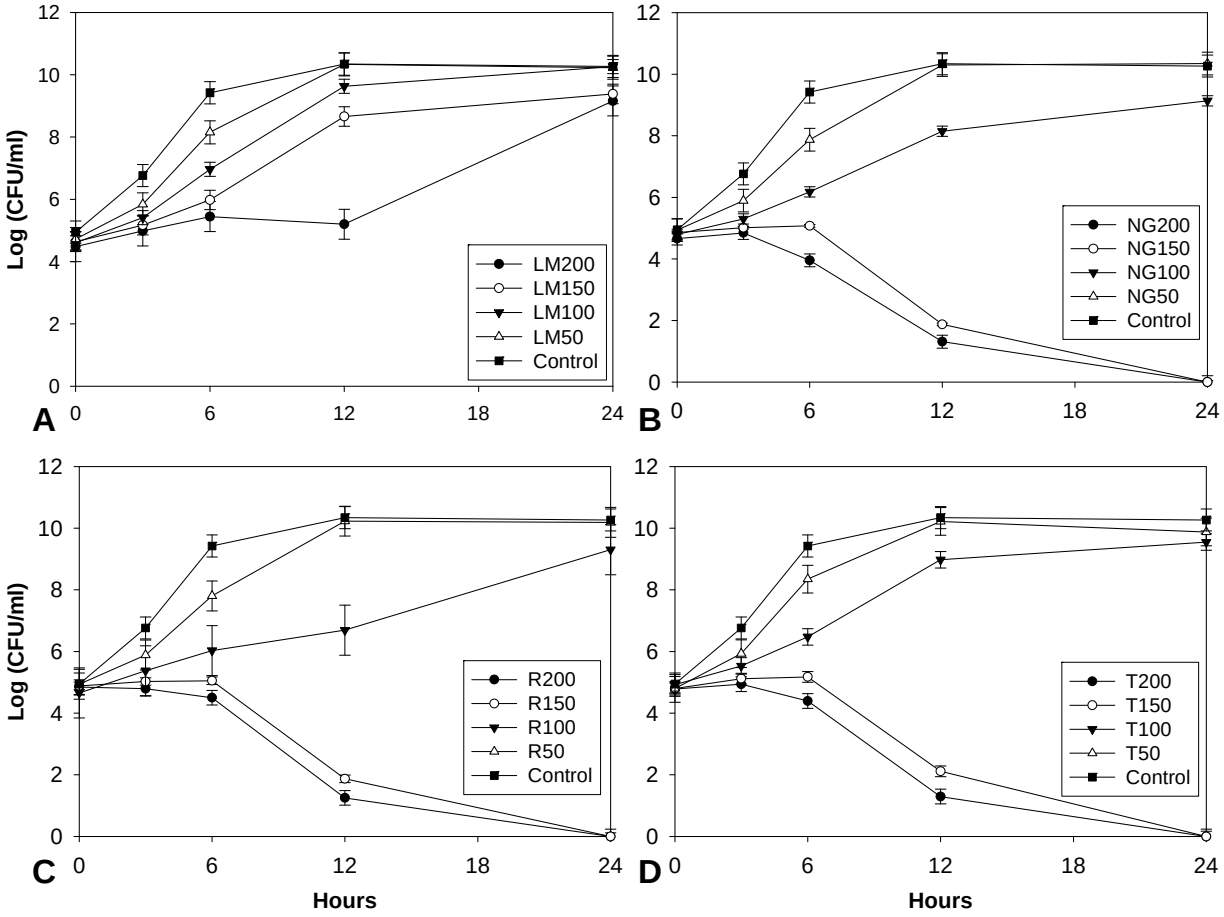

Figure 3-3. Antimicrobial activity of Yerba Mate tea, (A) LM: La Mulata, (B) NG: Nobleza Gaucha, (C) R: Rosamonte and (D) T: Taragui, aqueous extracts at 200, 150, 100 and 50 μg ml^{-1} against *Escherichia coli* O157:H7 ATCC 43894. Error bars represent 95% confidence intervals using least significant differences ($P<0.10$).

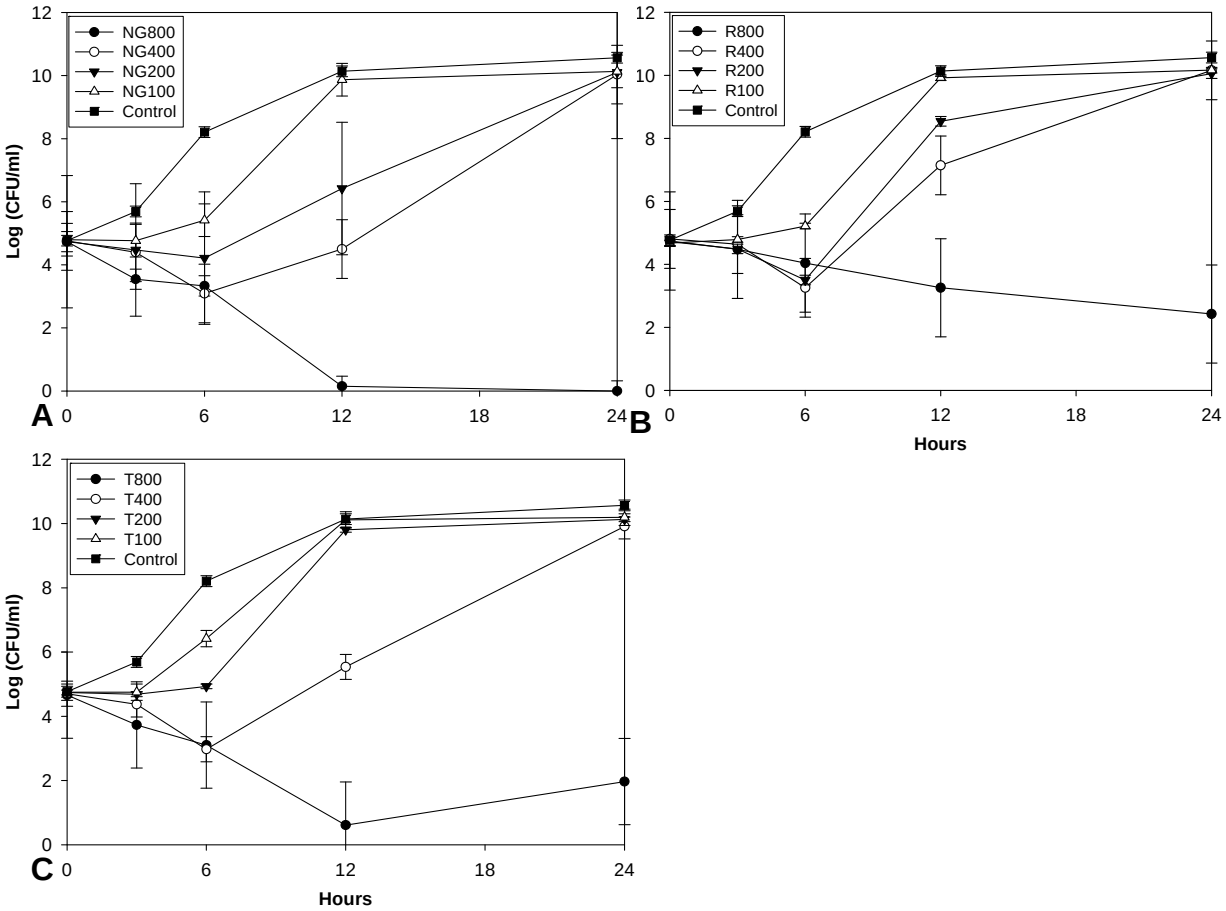

Figure 3-4. Antimicrobial activity of Yerba Mate tea, (A) NG: Nobleza Gaucha, (B) R: Rosamonte and (C) T: Taragui, aqueous extracts at 800, 400, 200 and 100 μg ml^{-1} against *Escherichia coli* O157:H7 'Cider'. Error bars represent 95% confidence intervals using least significant differences ($P<0.10$).

Chapter 4: Aqueous extracts of Yerba Mate (*Ilex paraguariensis*) as a natural antimicrobial against *Escherichia coli* O157:H7 in microbiological media and apple juice

Adapted from:

Kellie P. Burris, P. M. Davidson, C. Neal Stewart, Jr., S. Zivanovic and F. M. Harte. Aqueous extracts of Yerba Mate (*Ilex paraguariensis*) as a natural antimicrobial agent against *Escherichia coli* O157:H7 in microbiological media and apple juice. Journal Food Protection. Submitted.

I designed, executed and analyzed the experiments and wrote the manuscript.

4.1 Abstract

Ilex paraguariensis is popularly used in the preparation of a tea infusion (Yerba Mate), most commonly produced and consumed in the South American countries of Uruguay, Paraguay, Argentina and Brazil. In this study, aqueous extracts of commercial Yerba Mate tea were evaluated for their ability to inhibit or inactivate *Escherichia coli* O157:H7. Dialyzed, lyophilized aqueous extracts were screened for antimicrobial activity against *E. coli* O157:H7 strains ATCC 43894 and 'Cider' in tryptic soy broth (TSB) and pH-adjusted apple juice. A mixture of the two strains was used as the inoculum when apple juice was used as the medium. Minimum bactericidal concentrations were determined to be ca. 5 mg/ml and 10 mg/ml for ATCC 43894 and 'Cider', respectively in TSB. Higher concentrations of the extract were required to inactivate *E. coli* O157:H7 in modified apple juice. An approximate 4.5 log reduction was observed for *E. coli* O157:H7 treated with 40 mg/ml extract. It was concluded that aqueous extracts from commercial Yerba Mate have potential to be used as antimicrobials in foods and beverages against foodborne pathogenic bacteria.

Key words: Yerba Mate, *Ilex paraguariensis*, antimicrobial activity, *E. coli* O157:H7, apple juice

4.2 Introduction

Increasing incidence of foodborne illnesses along with a consumer demand for more natural foods have prompted the need for novel antimicrobials. Foodborne illnesses are a continuous threat to public health and Scallan et al. (2011) estimate that the 31 major foodborne pathogens account for nearly 9.4 million people becoming sick, more than 55,961 hospitalizations, and 1,351 deaths from foodborne illness each year in the United States with an estimated economic cost of $152 billion annually (Scharff, 2010).

Escherichia coli O157:H7 is a toxin-producing enteropathogen responsible for a hemorrhagic form of colitis, bloody diarrhea and hemolytic uremic syndrome. Apple juice which is a high acid food (pH <4.6) was believed at one time to be inhibitory to the survival and growth of *E. coli* O157:H7 (Marques and Farah, 2009). However, numerous foodborne illness outbreaks have been associated with the consumption of high acid, unpasteurized fruit juices (Cody et al., 1999). Regulations were developed in 2001 (21 CFR 120) that require treatments to achieve a 5-log reduction of *E. coli* O157:H7 in fruit juices. Traditionally, weak organic acids have been added as preserving agents in juices; however, consumers are less accepting of perceived "chemical additives" as preservatives and desire more natural products. This shift in consumer acceptance has pressed the food industry to examine more natural sources of antimicrobials, such as from botanicals.

Teas, mainly from the species *Camellia sinensis* contain components that provide antimicrobial activity against a variety of Gram-negative and Gram-positive foodborne pathogens (Diker et al., 1991; Kubo et al., 1993; Yam et al., 1997; Rasheed and Haider, 1998; Sakanaka et al., 2000;

Taguri et al., 2004; Yoda et al., 2004; Bandyopadhyay et al., 2005; Si et al., 2006; Almajano et al., 2008; Juneja et al., 2009 a,b). However, little research has been done on the use of Yerba Mate tea as a food antimicrobial. Yerba Mate is a popular tea infusion from the perennial botanical, *Ilex paraguariensis*, and is traditionally found, commercially produced and primarily consumed in the South American countries, Paraguay, Uruguay, Brazil and Argentina. With a demand for more healthy and natural foods, this tea is quickly increasing in popularity in the United States (Heck et al., 2008) While a variety of medicinal and pharmacological properties for Yerba Mate have been characterized (Gugliucci and Stahl, 1995; Carini et al., 1998; Filip et al., 2000; Andersen and Fogh, 2001; Gorzalczany et al., 2001; Mazzotta, 2001; Gorgen et al., 2005; Anesini et al., 2006; Bastos et al, 2006; Bastos, 2007; Bastos et al., 2007; Filip et al., 2009), limited research has been conducted on its effectiveness as an antimicrobial (Kubo et al., 1993; Hongpattarakere, 2000; Rauha et al., 2000; Si et al., 2006; Burris et al., 2011) and even less for its ability to preserve foods (Panizzi et al., 2002) and beverages, such as apple juice.

Typically, plant extracts that have been used as antimicrobial food preservatives have a negative sensory effect on the flavors and odors of the food. The flavor of Yerba Mate has been described as bitter, acid, astringent, hay, green, humid, toasted and paper (Cruz et al., 2003). While these terms seem negative, a study incorporating Yerba Mate into precooked chicken meat balls resulted in no effect on the taste or smell of the food product (Panizzi et al., 2002), indicating potential sensory acceptance in foods.

The goal of this study was to determine the effectiveness of Yerba Mate aqueous extracts against *E. coli* O157:H7, one of the most common pathogens associated with outbreaks in apple juice, in optimal growth conditions.

4.3 Materials and Methods

4.3.1 Culture preparation

E. coli O157:H7 ATCC 43894 and strain 'Cider' were stock cultures obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville. All cultures were grown in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) and stored at -20°C in glycerol. Working cultures were obtained by inoculating 50 ml TSB with 100 µl stock cultures and incubating for 24 h at 35-37°C. After incubation, the cultures were diluted to ca. 5.0-6.0 log CFU/ml and tested for antimicrobial activity.

4.3.2 Aqueous extraction

Dried leaves of a single commercial brand of Yerba Mate (Taragui, Argentina) were purchased from a local international supermarket. Dried tissue was finely ground (<1 mm) with a coffee grinder (Braun) for 15-20 s. Extracts were obtained by adding sterile water at a ratio of 3.6 ml to 1 g ground tissue, were allowed to stand for 2 h at 4 °C with occasional mixing to maximize extraction and were subsequently centrifuged at 5000 x g for 30 min to remove larger particles. Aqueous extracts were then dialyzed against deionized water for 36 h using a 3500 MWCO SnakeSkin® Pleated Dialysis Tubing (Pierce Biotechnology, Rockford, IL) to remove low molecular weight compounds. Resulting extracts were centrifuged at 5000 x g for 30 min to

remove sediment and frozen at -80 °C. Frozen extracts were then lyophilized using VirTis AdVantage Plus BenchTop freeze dryer (SP Industries, Gardiner, NY). This process was repeated until a sufficient quantity of lyophilized extract had been collected to allow for the antimicrobial assays. Lyophilized extracts were stored at room temperature in a sealed container.

4.3.3 Phenolic determination

Lyophilized extract (25 mg) was resuspended in 5 ml 95% ethanol to a final concentration of 5 mg/ml, filtered through Whatman No. 4 and measured for total phenolics. Total phenolics were quantified spectrophotometrically at 725 nm using Folin–Ciocalteau reagent (Montreau, 1972) with caffeic acid as the standard. The result is the mean value of nine replications.

4.3.4 Time kill assays

Dialyzed, lyophilized aqueous extracts (0-1000 mg) were diluted in 10 ml sterile water and filter sterilized using 0.22 mm (Millipore). Diluted extracts (10 ml) were mixed with bacteria harvested at late logarithmic phase and diluted to ca. 5.0-6.0 log CFU/ml. Bacteria and extracts were incubated in TSB and apple juice (Laura Lynn brand, Ingles Foods, pH adjusted to 6.0) at 35-37°C and at regular intervals (0, 3, 6, 12 and 24 h) a bacterial suspension sample was collected, serially diluted in 0.1% peptone, plated in duplicate using tryptic soy agar (TSA; Becton Dickinson), incubated for 24 h at 35-37°C, and then CFU enumerated. All experiments were duplicated and average values were reported. MIC and MBC were determined for each strain in TSB and for the combined strains in apple juice. MIC was determined as the lowest concentration tested that did not demonstrate bacterial growth (inhibition) above the original

inoculums ca. 5.0-6.0 log CFU/ml after 24 h. The MBC was determined as the lowest concentration tested where bacterial death (inactivation) was observed after 24 h.

4.3.5 Statistical analysis

Data were analyzed as a completely randomized design with four replicates. Analysis of variance (ANOVA) was done using the general linear model procedure of SAS (version 9.2, SAS Institute, Cary, NC). Least significant differences (LSD) were used to compare treatment mean values when significant differences ($p < 0.05$) were found. Error bars represent 95% confidence intervals for the mean using LSD.

4.4 Results and discussion

We obtained ca. 12 g lyophilized extract from the extraction and processing of 280 g commercial Yerba Mate tea (Taragui, Argentina). Quantitative analysis of the phenolics in the rehydrated aqueous extracts (5 mg lyophilized extract/ml) yielded ca. 79 μg caffeic acid equivalent/ml. Based upon this assay, for every g lyophilized extract or g of tea, represented ca. 16 mg or 0.8 mg respectively. This is similar to results found by Filip and others (2001), with caffeic acid content 0.023% of dried weight or 23 mg of caffeic acid/mg dried weight. However, Mazzafera (1997) determined caffeic acid content as 340 mg for every serving of tea (50-60 g) brewed in the traditional manner. This caffeic acid content was nearly 20 times higher than what we found, most likely due to preparation and/or brand. In comparison, ground coffee has been shown to have a total phenolic content of 52.5-57 mg gallic acid equivalents/g (Lakenbrink et al., 2000).

The dialyzed, lyophilized aqueous extracts of Yerba Mate were evaluated for their ability to inhibit growth of and/or inactivate *E. coli* O157:H7 in microbiological media and apple juice (pH adjusted to 6.0). Lyophilized extracts were diluted in 10 ml sterile water and tested against *E. coli* O157:H7 at 0-20 mg/ml for microbiological media and 0-40 mg/ml for modified apple juice. Processed extracts derived from commercially available Yerba Mate were effective at inhibiting and inactivating both strains of *E. coli* O157:H7 tested in microbiological media and modified apple juice. (Fig. 4-1 and 4-2). Activity was observed using initial tea quantities of less than or equal to typical consumption levels (50 g) with activities occurring at 3 and 6 g dried tea equivalent for *E. coli* O157:H7 ATCC 43894 and 'Cider' in microbiological media (Fig. 4-1), respectively, and 23 g dried tea equivalent for *E. coli* O157:H7 mixed strains in modified apple juice (Fig. 4-2). Higher concentrations were required to inhibit and inactivate *E. coli* O157:H7 in modified apple juice as compared to microbiological media.

MICs were determined to be 5 mg/ml for both strains of *E. coli* O157:H7 in microbiological media (Fig. 4-1A,B). MBCs were determined to be 5 mg/ml for *E. coli* O157:H7 strain ATCC 43894 and 10 mg/ml for *E. coli* O157:H7 strain 'Cider' in microbiological media (Fig. 4-1A,B). An approximate 4.5 log reduction was observed for *E. coli* O157:H7 treated with 40 mg/ml extract in modified apple juice which is near to the requirement of 21 CFR120.

An aqueous extraction protocol for Yerba Mate was used to obtain extracts with antimicrobial activity against *E coli* O157:H7. Crude extracts and many individual compounds in the more consumed green and black teas have been extensively studied for broad antimicrobial activity (Kubo et al., 1992; Yam et al., 1997; Taguri et al., 2004; Yoda et al., 2004; Si et al., 2006).

However, limited research has been conducted on extracts and compounds possessing antimicrobial activity derived from Yerba Mate (Kubo et al., 1993; Schenkel et al., 1996; Hongpattarkere, 2000; Sari et al., 2007; Tsai et al., 2008; Burris et al., 2011). While many of the compounds found in Yerba Mate extracts are known (Kubo et al., 1993; Hongpattarakere, 2000; Filip et al., 2001; Heck and de Mejia, 2007), the identification of those contributing to antimicrobial activity and whether they have combined additive or synergistic effects are not entirely known. Caffeine, ursolic acid and chlorogenic acid are three common compounds that have been identified from Yerba Mate; however, none demonstrated antimicrobial activity against *E. coli* at 400 μg/ml (Kubo et al., 1993), a concentration significantly lower than tested here. Other compounds isolated from Yerba Mate include the polyphenols, caffeic acid, caffeoyl derivative, caffeoylshikimic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin and theobromine (Heck and de Mejia, 2007; Marques and Farah, 2009) which may provide antimicrobial activity against *E. coli* O157:H7. In pure form, caffeic and chlorogenic acids have demonstrated activity against Gram-negative bacteria (Herald and Davidson, 1983; Puupponen-Pimia et al., 2001). Furthermore, several of the flavonols found in Yerba Mate have been examined for their antimicrobial activity—kaempferol, quercetin, and rutin (Rauha et al., 2000; Panizzi et al., 2002). However, in previous studies, neither kaempferol nor quercetin were found to inhibit *E. coli* (Puupponen-Pimia et al., 2001; Panizzi et al., 2002). N-hexane distillate extracts of Yerba Mate have been shown to be effective antimicrobial agents (Kubo et al., 1993). Ten main compounds were identified in the N-hexane distillate extracts of Yerba Mate and include linalool, α-ionone, β-ionone, α-terpineol, octanoic acid, geraniol, 1-octanol, nerolidol, geranylactetone and eugenol (Kubo et al., 1993). These distillate compounds tested were only weakly active against *E. coli* (Kubo et al., 1993). Compounds present in our aqueous extracts

demonstrating antimicrobial activity may be different than those reported by Kubo et al. (1993) since we utilized a water-based extraction. Further, we determined inhibition and inactivation of *E. coli* O157:H7 whereas Kubo et al. (1993) and Sari et al. (2007) only demonstrated weak and no activity respectively against *E. coli*. Perhaps one or more of the identified compounds are acting in concert and contributing to the antimicrobial activity against *E. coli* O157:H7.

While in this study we found that aqueous extracts from Yerba Mate provide antimicrobial activity against *E. coli* O157:H7 at a relatively low level (5-10 mg/ml), Schenkel et al. (1996) determined that neither a crude ethanolic extract (100 mg/ml) nor saponin fraction (100 mg/ml) from Yerba Mate demonstrated antimicrobial activity against *E. coli*. Hongpatarakere (2000) determined the concentration of aqueous methanolic crude extract required to inhibit *E. coli* O157:H7 was 150 mg/ml and no observable activity was detected at 30-60 mg/ml. Here we demonstrated activity at much lower concentrations (5-10 mg/ml) for complete inactivation of two strains of *E. coli* O157:H7.

Typically higher concentrations of antimicrobial are required in food systems to achieve the same levels of bacterial inactivation, due to interaction of food components with antimicrobial compounds. In the present study, ca. 4-8 times more lyophilized extract was required in modified apple juice than in microbiological media. However, even in a food system, less extract was required than that used by Hongpatarakere (2000) to inactivate and achieve an almost 5-log reduction in *E. coli* O157:H7.

One chief goal of the food and beverage industry is to protect consumers from the harmful effects of foodborne pathogens. Dialyzed, lyophilized aqueous extracts of Yerba Mate demonstrated bactericidal activity against *E. coli* O157:H7 in microbiological media as well as an additive in a beverage system. Results demonstrated that relatively low concentrations of Yerba Mate aqueous extracts provide antimicrobial activity against *E. coli* O157:H7 in modified apple juice. Consumer demand for natural products has prompted researchers to find natural sources of antimicrobials for use in foods as alternatives or in addition to currently used regulated antimicrobials. Here it was demonstrated that the antimicrobial activity of a bio-based antimicrobial, Yerba Mate extract, could be used in beverages for protection against *E. coli* O157:H7.

4.5 Acknowledgements

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Appendix

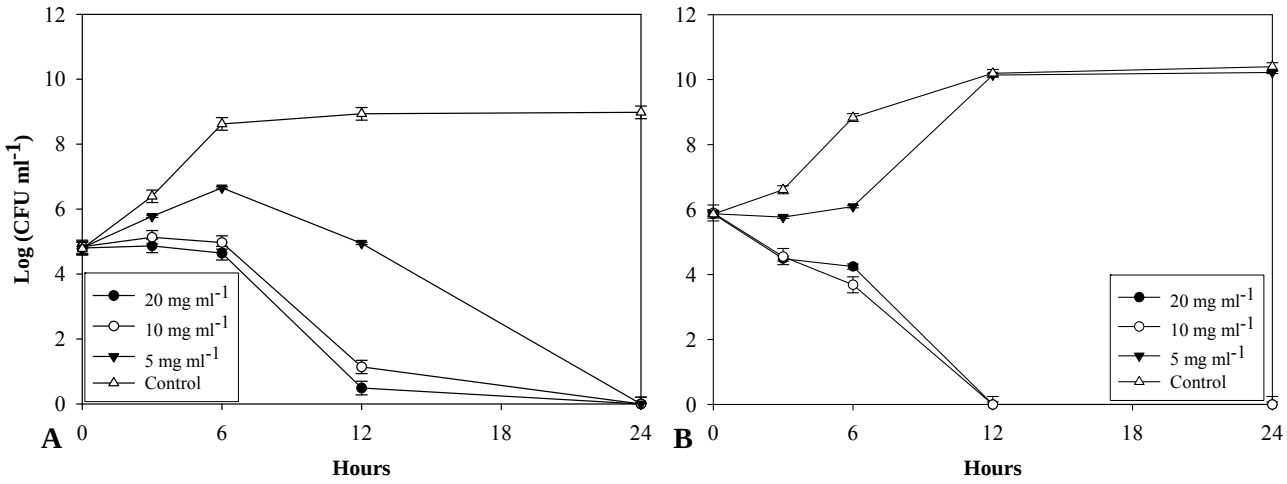

Figure 4-1. Antimicrobial activity of Yerba Mate (Taragui) extracts at 0, 5, 10, and 20 mg/ml against *Escherichia coli* O157:H7 (A) strain ATCC 43894 and (B) strain 'Cider' in tryptic soy broth. Error bars represent 95% confidence intervals for the mean using least significant differences (P<0.05).

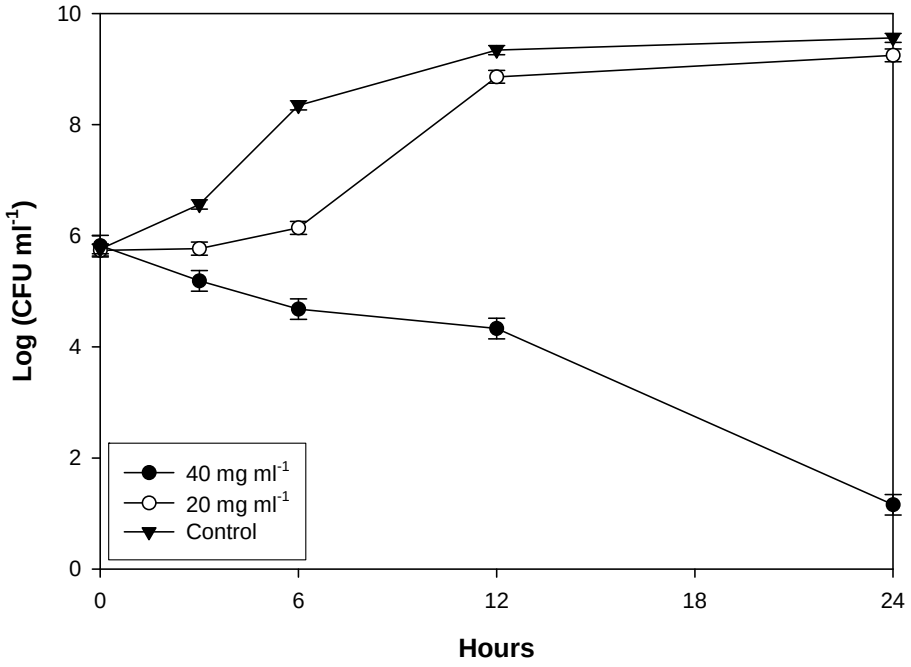

Figure 4-2. Antimicrobial activity of Yerba Mate (Taragui) extracts at 0, 20, and 40 mg/ml against a mixture (1:1) of *Escherichia coli* O157:H7 strains ATCC 43894 and 'Cider' in modified apple juice. Error bars represent 95% confidence intervals for the mean using least significant differences ($P<0.05$).

Chapter 5: Killing methicillin-resistant staphylococci with tea

5.1 Abstract

Staphylococci are important opportunist pathogens affecting human and animal health. The most significant species are *Staphylococcus aureus* and *Staphylococcus pseudintermedius*, which have developed methicillin-resistance, making them a serious public health concern. While the best method of combating these organisms currently is through the use of non-beta-lactam antibiotics, the concern remains that they will develop resistance to all currently used antibiotics. We present an alternative source for a natural methicillin-resistant *S. aureus* (MRSA) antibiotic: tea; more specifically aqueous extracts from a tea plant *Ilex paraguarensis*, Yerba Mate. Methicillin-resistant staphylococci were eliminated after 12 hr incubation.

5.2 Brevia article

Antibiotic resistant bacteria are a great concern to human and animal health. With the first reports of methicillin-resistance bacteria occurring in the 1970s, antibiotic resistance has emerged as a significant problem and staphylococci are the bacterial genus at the forefront of developed resistance. In the past, the primary antibiotic to manage methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudintermedius* (MRSP) infections has been vancomycin; however, recently, these bacteria have developed a reduced susceptibility or increased resistance to the antibiotic (Hiramtsu, 2001). As in MRSA, the methicillin resistance of *S. pseudintermedius* is mediated by the *mecA* gene, which encodes for the production of the penicillin-binding protein 2A, a protein with a low affinity for beta-lactams (Bemis et al., 2009). MRSP has emerged as a recent problem, and there seems to be two multilocus sequencing types that are predominant: ST68 in the United States and ST71 in Europe (Black et al., 2009). With these increasing variants of resistance, the need for alternative compounds to manage these bacteria is required.

Plants have been used as a source of bioactive agents for thousands of years. Of the 252 drugs considered essential by the World Health Organization, 11% derived from flowering plants (Rates, 2001). A recent focus has been to obtain new bioactive compounds from botanicals for use in a variety of industries is aimed at the elimination and/or inhibition of pathogenic bacteria for crops, medicines, cosmetics, and animal/human feeds/foods. Yerba Mate, a popular tea beverage, from the plant *Ilex paraguarensis*, frequently consumed in the South American countries of Brazil, Uruguay, Paraguay and Argentina, is known for its antioxidant, anticancer, anti-inflammation, and anti-atherosclerosis qualities (reviewed in Heck and de Mejia, 2007).

Previous research has demonstrated the use of aqueous Yerba Mate extracts to kill methicillin-susceptible *S. aureus* (Burris et al., 2011; Hongpattarakere, 2000).

Due to the increased incidences of antibiotic resistant bacteria, we chose to examine the effectiveness of our aqueous plant-derived extracts against two strains each of the methicillin-resistant pathogens, MRSP, a clinically associated infection in dogs and cats, and MRSA.

Commercially available Yerba Mate (Taragui brand; Argentina) was subjected to aqueous extraction to obtain active extracts (see section 5.3). Reconstituted extracts were evaluated for antimicrobial activity against MRSA and MRSP using a microbiological medium. Initial bacterial counts were ca. 6 log CFU ml^{-1}. We demonstrated antimicrobial effectiveness of aqueous Yerba Mate extracts after 24 hr at 1 and 2 mg ml^{-1} against both strains of MRSP (Fig. 5-1) and MRSA (Fig. 5-2). An approximate >5 log reduction was observed in all strains at all concentrations after 24 hr (Fig. 5-1 and 5-2). MRSA strains appeared more susceptible to the extract than MRSP strains. However, complete inhibition of MRSA (Fig. 5-2) and MRSP (Fig. 5-1) strains occurred after only 12 hr at 8 mg ml^{-1}.

By demonstrating the effectiveness of Yerba Mate extracts against methicillin-resistant strains of pathogenic bacteria, we provide an alternative natural botanical to reduce and eliminate populations of antibiotic-resistant bacteria as well as an alternative or adjunct to current or ineffective antibiotics.

5.3 Materials and methods

5.3.1 Culture preparation

Staphylococcus pseudintermedius strains were stock cultures obtained from Dr. David Bemis from the College of Veterinary Medicine at the University of Tennessee, Knoxville and *S. aureuss* was purchased from American Type Culture Collection (ATCC). All cultures were grown in tryptic soy broth (TSB; Difco, Sparks, MD) and stocks stored at -20°C in glycerol. Working cultures were obtained by inoculating 50 ml TSB with 100 µl stock cultures and incubating for 24 hr at 35-37°C. After incubation, the cultures were diluted to ca. 5.0-6.0 log CFU (colony forming units) ml^{-1} and tested for antimicrobial activity.

5.3.2 Aqueous extraction

Dried leaves of a single commercial brand of Yerba Mate (Taragui; Argentina; *Ilex paraguariensis*) were purchased from a local international supermarket. Dried tissue was finely ground to a particle size <1 mm using a coffee grinder (Braun) for 15-20 s. Extracts were obtained by adding sterile deionized water at a ratio of 3.6 ml to 1 g ground tissue, were allowed to stand for 2 hr at 4 °C with occasional mixing to maximize extraction and were subsequently centrifuged at 5000 x g for 30 min. Aqueous extracts were then dialyzed at 4 °C against deionized water for 36 h using a 3500 MWCO SnakeSkin® Pleated Dialysis Tubing (Pierce Biotechnology, Rockford, IL) to remove low molecular weight compounds. Resulting extracts were centrifuged at 5000 x g for 30 min to remove sediment and frozen at -80 °C. Frozen extracts were then lyophilized using VirTis AdVantage Plus BenchTop freeze dryer (SP Industries, Gardiner, NY). This process was repeated until a sufficient quantity of lyophilized

extract had been collected to allow for the antimicrobial assays. Lyophilized extracts were stored at room temperature in a sealed container.

5.3.3 Time kill assays

Lyophilized extracts (0-300 mg) were diluted in 10 ml sterile water and filter sterilized using 0.22 μm (Millipore), mixed with bacteria (diluted to ca. 5.0-6.0 log CFU ml^{-1}) harvested at late logarithmic phase. Bacteria and extracts were incubated in TSB at 35-37°C and at regular intervals (0, 3, 6, 12 and 24 hr) a bacterial suspension sample was collected, serially diluted in 0.1% peptone, plated in duplicate using tryptic soy agar (TSA; TSB, Becton, Dickinson and Company, Sparks, MD and Agar, Fisher Scientific, USA), incubated for 24 hr at 35-37°C, and then enumerated. All experiments were duplicated and average values were reported. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of extract tested that did not allow bacterial growth (inhibition) above the original inoculums of ca. 5.0-6.0 log CFU ml^{-1} after 24 hr. The minimum bactericidal concentration (MBC) was defined as the lowest concentration tested where bacterial death (inactivation) was observed after 24 hr.

5.3.4 Statistical analysis

Data were analyzed as a completely randomized design with four replicates by analysis of variance (ANOVA) using the general linear model (SAS 9.2, SAS Institute, Cary, NC). Least significant differences (LSD) were used to compare treatment mean values when significant differences (at the 0.05 probability level) were found. Error bars represent 95% confidence intervals for the mean using LSD.

5.4 Acknowledgements

This research was supported by the Ivan Racheff Chair of Excellence endowment and the Tennessee Agricultural Experiment Station. We especially thank Drs. David Bemis and Chad Black, College of Veterinary Medicine, University of Tennessee, Knoxville, for providing the MRSP samples for testing.

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Appendix

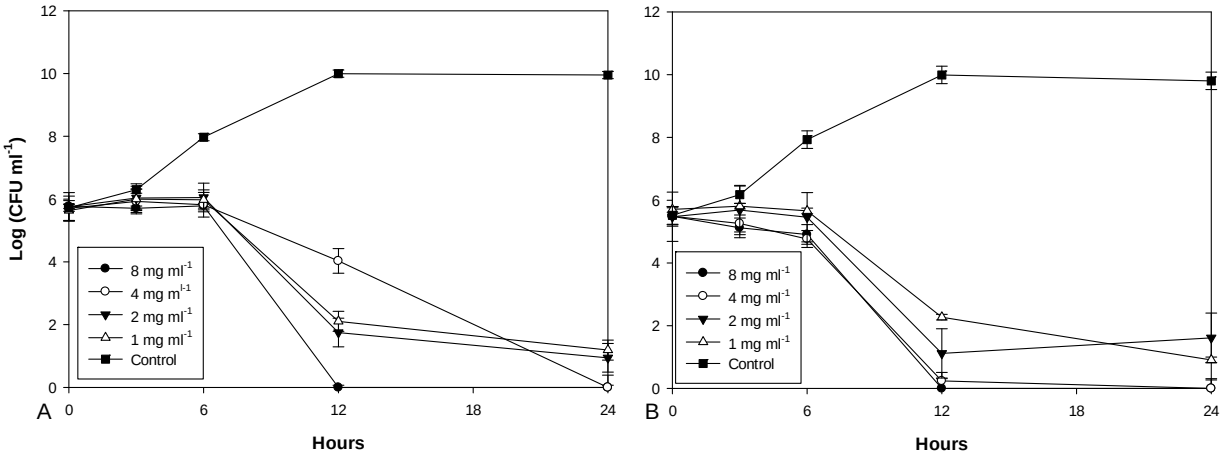

Figure 5-1. Antimicrobial activity of Yerba Mate (Taragui) extracts at 0, 1, 2, 4, and 8 mg ml^{-1} against methicillin-resistant *Staphylococcus pseudintermedius* (A) F13-2 and (B) H3-74. Error bars represent 95% confidence intervals using least significant differences ($P<0.05$).

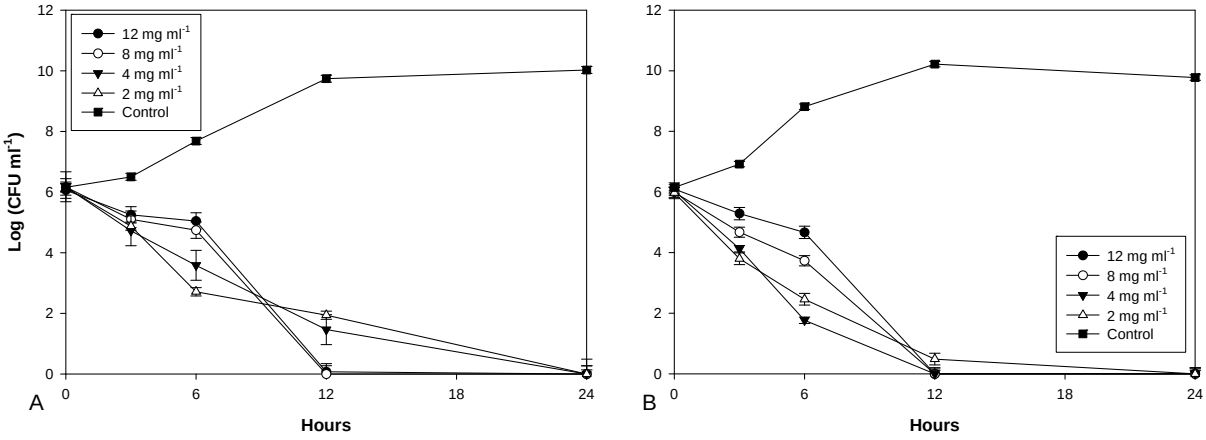

Figure 5-2. Antimicrobial activity of Yerba Mate (Taragui) extracts at 0, 2, 4, 8 and 12 mg ml^{-1} against methicillin-resistant *Staphylococcus aureus* (A) ATCC 33591 and (B) ATCC 33593. Error bars represent 95% confidence intervals using least significant differences ($P<0.05$).

Chapter 6: Antimicrobial activity of Yerba Mate (*Ilex paraguariensis*) against *Pseudomonas syringae* pv. tomato

6.1 Abstract

The use of natural products to control disease in tomatoes and other food crops is becoming increasingly important to producers, particularly in organic production. *Ilex paraguariensis* is used in the preparation of a widely popular tea beverage in South America in the countries of Uruguay, Paraguay, Argentina and Brazil. Here we examined this commercial tea (Taragui, Argentina), derived from the plant, *I. paraguariensis,* for its ability to possess compounds that are effective against plant pathogenic bacteria for use in crop protection. Dialyzed, lyophilized aqueous extracts from commercially available Yerba Mate tea were produced. Extracts were screened for antimicrobial activity against *Pseudomonas syringae* pv. tomato *in vitro*. Extracts at 4 mg/ml demonstrated antimicrobial activity against *P. syringae* pv. tomato with initial bacterial concentrations of approximately 3 and 6 log CFU/ml. Complete inhibition of *P. syringae* pv. tomato was observed after 8 hr and no recovery of the bacteria was observed after 48 hr. We aim to develop a natural antimicrobial for use in the horticultural industry that is bactericidal or inhibitory to a broad spectrum of plant pathogenic microorganisms.

Key words: Aqueous extracts, Yerba Mate, plant pathogens, tomato, bacterial speck disease

6.2 Introduction

Plants have been used as a source of bioactive agents for thousands of years. Plants are constantly attacked by a variety of pathogens and have adapted natural defense mechanisms to help protect them from disease. *Ilex paraguariensis*, a native South American holly shrub from the Aquifoliaceae family, is mainly produced and consumed in the countries of Argentina, Brazil, Paraguay, and Uruguay (Grigioni et al., 2004). Yerba Mate is the commercial product derived from the dried leaves and stems of *I. paraguariensis* and has been recognized for a variety of nutritional and medicinal benefits.

Several compounds have been identified from the leaves of *Ilex paraguariensis* and include an assortment of polyphenols, xanthines, caffeoyl and chlorogenic acid derivatives, saponins, terpenes, flavonoid aglycones, flavonoid glycosides, and minerals (Alikaridis, 1987; Gosmann and Schenkel, 1989; Carini et al., 1998; Clifford and Ramirezmartinez, 1990; Filip et al., 2001; Bastos et al., 2006; Bastos et al., 2007; Bravo et al., 2007; Cardozo et al., 2007; Heck and de Mejia, 2007; Marques and Farah, 2009). Studies have suggested that consuming Yerba Mate leaves may provide antioxidant (Gugliucci and Stahl, 1995; Carini et al., 1998; Filip et al., 2000; Anesini et al., 2006; Bastos et al., 2006; Bastos et al., 2007; Pagliosa et al., 2010), antiobesity (Andersen and Fogh, 2001), antidiabetic (Lunceford and Gugliucci, 2005), diuretic (Gorgen et al., 2005), chemopreventative, antifungal (Filip et al., 2010), stimulant (Athayde et al., 2000; Filip et al., 1998) and digestive aid (Gorzalczany et al., 2001) properties. While Yerba Mate has been recognized for these potential pharmacological activities, little research has been conducted on its antimicrobial properties (Kubo et al., 1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et

al., 2008; Burris et al. 2011) and the use of Yerba Mate extracts for crop protection is fairly novel (Racanicci et al., 2009; Burris et al., 2011).

Known compounds have been tested for antimicrobial activity against rather few microorganisms. Kubo et al. (1993) determined that the 10 major volatile constituents (linalool, a-ionone, b-ionone, a-terpineol, octanoic acid, geraniol, l-octanol, nerolidol, geranylacetone, and eugenol) identified by GC-MS analysis in Yerba Mate tea were effective against *Streptococcus mutans*. Crude extracts and a variety of isolated compounds derived from Yerba Mate have been shown active against a broad spectrum of Gram-positive and Gram-negative bacteria (Kubo et al., 1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et al., 2008; Burris et al., 2011). This points to possible use of crude extracts and isolated compounds as novel antimicrobials in crop protection.

The goal of this study was to determine the effectiveness of Yerba Mate aqueous extracts against *P. syringae* pv. tomato, a common pathogen associated with tomatos causing bacterial speck disease. The antimicrobial activity of these plant extracts may be attributed to a variety of different compounds and may provide an alternative organic antibacterial or may be used in conjunction with current bactericidal agents.

6.3 Materials and methods

6.3.1 Culture Preparation

Pseudomonas syringae pv. tomato was obtained from the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville. Cultures were grown in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) and stored at -20 °C. Working cultures were obtained by inoculating 50 ml TSB with 100 ml stock cultures and incubating for 24 hr at 30 °C. After incubation, cultures were diluted to ca. 3.0 or 6.0 log CFU/ml and tested for antimicrobial activity.

6.3.2 Total aqueous extraction

Dried leaves of a single commercial brand of Yerba Mate (Taragui, Argentina) were purchased from a local international supermarket. Dried tissue was finely ground (<300 μm) with a coffee grinder (Braun) for 15-20 sec. Extracts were obtained by adding sterile water at a ratio of 3.6 ml to 1 g ground tissue, were allowed to stand for 2 hr at 4 °C with occasional mixing to maximize extraction and were subsequently centrifuged at 5000 x g for 30 min to remove larger particles. Aqueous extracts were then dialyzed against deionized water for 36 h using a 3500 MWCO SnakeSkin® Pleated Dialysis Tubing (Pierce Biotechnology, Rockford, IL) to remove low molecular weight compounds. Resulting extracts were centrifuged at 5000 x g for 30 min to remove sediment and frozen at -80 °C. Frozen extracts were then lyophilized using VirTis AdVantage Plus BenchTop freeze dryer (SP Industries, Gardiner, NY). This process was

repeated until a sufficient quantity of lyophilized extract had been collected to allow for the antimicrobial assays. Lyophilized extracts were stored at room temperature in a sealed container.

6.3.3 Time kill assays

Dialyzed, lyophilized aqueous extracts (0-500 mg) were diluted in 10 ml sterile water and filter sterilized using 0.22 mm (Millipore). Diluted extracts (10 ml) were mixed with bacteria harvested at late logarithmic phase and diluted to ca. 3.0 log CFU/ml (low level) or 6.0 log CFU/ml (high level). Bacteria and extracts were incubated in TSB at 30°C and at regular intervals (0, 8, 24 and 48 h) a bacterial suspension sample was collected, serially diluted in 0.1% peptone, plated in duplicate using tryptic soy agar (TSA; Becton Dickinson), incubated for 48 h at 30°C, and then CFU enumerated. All experiments were duplicated and average values were reported. The MBC was determined as the lowest concentration tested where bacterial death (inactivation) was observed.

6.3.4 Statistical analysis

Data were analyzed as a completely randomized design with four replicates. Analysis of variance (ANOVA) was done using the general linear model procedure of SAS (version 9.2, SAS Institute, Cary, NC). Least significant differences (LSD) were used to compare treatment mean values when significant differences ($p < 0.05$) were found. Error bars represent 95% confidence intervals for the mean using LSD.

6.4 Results and discussion

The dialyzed, lyophilized aqueous extracts of Yerba Mate were evaluated for their ability to inhibit growth of and/or inactivate *P. syringae* pv. tomato in microbiological media. Lyophilized extracts were diluted in 10 ml sterile water and tested against *P. syringae* pv. tomato at 0-20 mg/ml, depending upon initial inoculum level. Processed extracts derived from commercially available Yerba Mate were effective at inactivating *P. syringae* pv. tomato in microbiological media. Activity was observed using initial tea quantities of less than or equal to typical consumption levels (50 g).

Antimicrobial activity was demonstrated at 2 mg/ml (Fig. 6-1) for low initial bacterial population and 4 mg/ml for high initial bacterial population (Fig. 6-2). Bacteria were reduced approximately > 3.0 log CFU/ml for the low initial bacterial population (Fig. 6-1) and approximately > 6.0 log CFU/ml for the high initial bacterial population (Fig. 6-2) by 8 hr. No bacterial recovery at the level of detection (< 1 CFU) was observed after 48 hr (Figs. 6-1, 6-2). Hongpattarakere (2000) determined that Yerba Mate extracts at 150-300 mg/ml were effective against *Pseudomonas fluorescens* (6.4-6.8 log CFU/ml), but not at levels ≤60 mg/ml. Here we determined activity against *P. syringae* at much lower levels (4 mg/ml) at similar initial inoculums (6.0 log CFU/ml) (Fig. 6-2).

Crude extracts and known compounds from Yerba Mate have been studied for antimicrobial activity against a variety of bacterial and fungal pathogens (Kubo et al., 1993; Hongpattarakere, 2000; Sari et al., 2007; Tsai et al., 2008; Filip et al., 2010; Burris et al., 2011); however, the use of Yerba Mate as an antimicrobial in foods and for crop protection is a relatively new idea

(Racanicci et al., 2009; Burris et al., 2011) and has not been fully researched. Many polyphenolic compounds have been identified from Yerba Mate and include caffeic acid, caffeine, caffeoyl derivatives, caffeoylshikimic acid, chlorogenic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin and theobromine (Heck and de Mejia, 2007; Marques and Farah, 2009) and may contribute to the antimicrobial activity against bacterial pathogens. Caffeoyl derivatives, methylxanthines, and rutin have been identified from Yerba Mate aqueous extracts and have shown antifungal activity (Filip et al., 2010). N-hexane extracts of Yerba Mate have demonstrated activity against the Gram-positive oral bacterium, *Streptococcus mutans* (Kubo et al., 1993). Ten major volatile compounds in these extracts have been identified as linalool, α-ionone, β-ionone, α-terpineol, octanoic acid, geraniol, 1-octanol, nerolidol, geranylactetone and eugenol (Kubo et al. 1993) and have been shown effective against a broad spectrum of Gram-positive and Gram-negative bacteria, at concentrations between 12.5 and 1600 $\mu g\ ml^{-1}$ (Taniguchi et al., 1978; Kubo et al., 1991, 1993; Sari et al., 2007). The Gram-positive bacteria, *Bacillus subtilis, Brevibacterium ammoniagenes, Propionibacterium acnes, S. aureus,* and *Streptococcus mutans,* and five fungi, *Saccharomyces cereuisiae, Candida utilis, Pityrosporum ovale, Penicillium chrysogenum,* and *Trichophyton mentagrophytes,* were inhibited by at least one of the 10 identified compounds by Kubo et al. (1993). However, none of the N-hexane extracts tested were effective against the Gram-negative bacteria, *Pseudomonas aeruginosa* or *Enterobacter aerogenes* and were only weakly active against *E. coli* (Kubo et al., 1993). Burris et al. (2011) determined that aqueous extracts from Yerba Mate demonstrated antimicrobial activity against *S. aureus* and *E. coli* O157:H7, providing evidence of antimicrobial activity against both Gram-positive and Gram-negative bacteria and the potential of activity coming from an unidentified compound. Caffeic and chlorogenic acids in their pure form have demonstrated

activity against Gram-negative bacteria (Herald and Davidson, 1983; Puupponen-Pimia et al., 2001). However, several researchers have found results contradictory to the activity being attributed to caffeic or chlorogenic acids (Kubo et al., 1993; Rauha et al., 2000). Kubo et al. (1993) determined that the three main compounds found in Yerba Mate, caffeine, ursolic acid and chlorogenic acid, were not effective against Gram-negative or Gram-positive bacteria, including *E. coli* and *S. aureus.* Rauha et al. (2000) also found that caffeic acid did not demonstrate antimicrobial activity against the Gram-positive bacteria, *S. aureus*, *S. epidermidis*, or *Bacillus subtilis.* Caffeine was shown not to be inhibitory to Gram-positive bacteria, *Streptococcus mutans* (Daglia et al., 2002), providing evidence that caffeine was not contributing to the antimicrobial activity observed by Kubo et al. (1993). Kaempferol demonstrated activity against *S. aureus* (Rauha et al., 2000), but did not to inhibit *S. epidermidis* (Rauha et al., 2000) or *E. coli* (Puupponen-Pimia et al., 2001) while quercetin exhibited strong inhibition against *S. aureus, S. epidermidis* and *B. subtilis* (Rauha et al., 2000). However, Panizzi et al. (2002) found that neither kaempferol nor quercetin demonstrated antimicrobial activity against *S. aureus* or *E. coli*. Caffeoylquinic acid derivatives have been shown to contribute to antimicrobial activity in other crude plant extracts (Chakraborty and Mitra, 2008) and have been found in Yerba Mate extract (Filip et al., 2000, 2010). The most likely scenario is that antimicrobial activity is provided by a combination of compounds, not just a single compound, found in Yerba Mate extracts. It is this combination that allows for antimicrobial activity against Gram-negative and Gram-positive bacteria, as observed by the ineffectiveness of activity by some individual compounds.

Our results demonstrated that relatively low concentrations of Yerba Mate aqueous extracts provide antimicrobial activity against *P. syringae* pv. tomato. The compounds responsible for antimicrobial activity appear to be relatively stable since these results were found using starting materials that had undergone commercial preparation and our extraction process which includes freeze drying. The use of natural plant products versus purified or synthesized chemicals as antimicrobials is becoming more popular for use in crops, particularly organic production.

We have utilized an aqueous extraction protocol to obtain extracts with antimicrobial activity against *Pseudomonas syringae* pv. tomato. We envision its potential for use in crop protection against plant pathogens, in seed production and for use in organic applications requiring alternative chemicals for the removal and inhibition of bacterial contamination and growth.

6.5 Acknowledgements

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Appendix

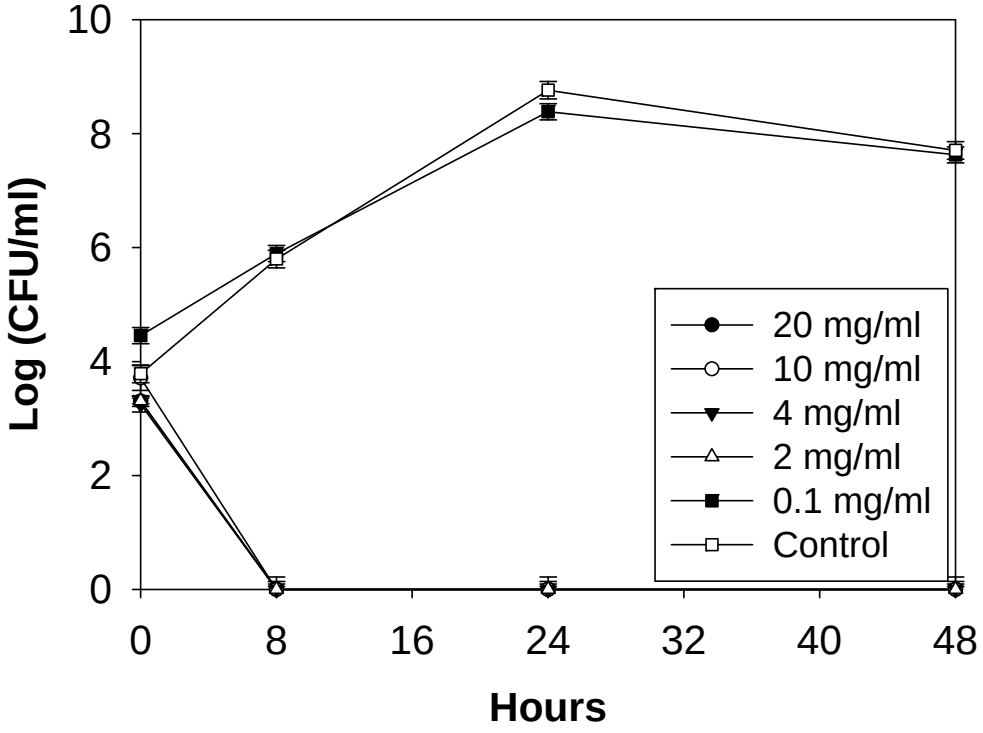

Figure 6-1. Antimicrobial activity of Yerba Mate (Taragui) extracts at 0, 2, 4, 10, and 20 mg/ml against *Pseudomonas syringae* pv. tomato with initial inoculum of ca. 3 log CFU/ml. Error bars represent 95% confidence intervals using least significant differences ($P<0.05$).

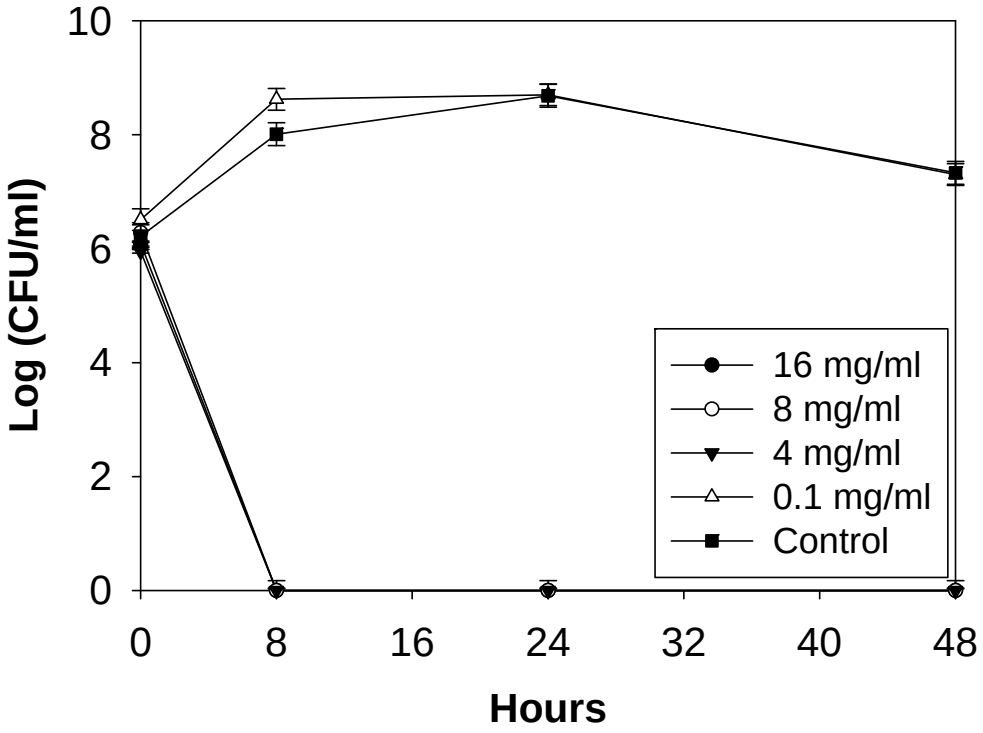

Figure 6-2. Antimicrobial activity of Yerba Mate (Taragui) extracts at 0, 4, 8, and 16g/ml against *Pseudomonas syringae* pv. tomato with initial inoculum of ca. 6 log CFU/ml. Error bars represent 95% confidence intervals using least significant differences ($P<0.05$).

Chapter 7: Conclusions

Plants have been used for thousands of years as sources of bioactive compounds for use in human health. However, within the last decade, the need for more natural and organic products has increased. Research on extracts and isolated compounds from Yerba Mate using to benefit human health has provided a number of potential pharmacological applications: antioxidant, antimicrobial, anti-inflammatory, antiobesity, and anticancer.

While many of the compounds found in Yerba Mate extracts are known, limited information is available on which compounds might contribute to antimicrobial activity and whether they may be additive or synergistic when used in combination. Here we have utilized an aqueous extraction protocol to obtain extracts with antimicrobial activity against Gram-positive and Gram-negative foodborne, human/animal and plant pathogens, *S. aureus*, *E. coli* O157:H7, methicillin-resistant *S. aureus* and *S. pseudintermedius*, and *P. syringae* pv. tomato.

Dialyzed, lyophilized aqueous extracts of Yerba Mate demonstrated bactericidal activity against Gram-positive and Gram-negative foodborne, human and/or plant pathogens. Our results demonstrated that relatively low concentrations of Yerba Mate aqueous extracts provide antimicrobial activity against Gram-positive and Gram-negative bacteria. Depending upon the pathogen tested, we obtained greater activity using less lyophilized extract.

Our compounds responsible for antimicrobial activity appear to be very stable since these results were found using starting materials that had undergone commercial preparation of blanching, drying, milling and aging and our extraction process, including lyophilization.

Initial tests against *S. aureus* and *E. coli* (Chapter 2) were performed with aqueous extracts concentrated using centrifugation (Spin-X columns, Corning, 5000 Da molecular weight cut-off) and used in antimicrobial assays based upon protein equivalents as determined by Bradford. We improved our process by lyophilizing our extracts and adding a direct amount of lyophilized extract for each treatment (Chapters 3-6), allowing for more adequate comparisons to previous studies.

We were able to demonstrate antimicrobial activity of aqueous Yerba Mate extracts in microbiological media and a food system, apple juice, against *E. coli* O157:H7. Negative sensory attributes are an important factor associated with the use of plant extracts as food preservatives. While Yerba Mate extracts have the potential to serve as food protectants, negatively associated sensory characteristics must be addressed for each food application.

While there exists the need for more research on isolation and identification of those compounds producing activity, evidence seems to show that Yerba Mate is a botanical with a variety of compounds that can be applied for use in human and plant health. Research confirms the influence cultivation and processing have on the chemical composition of Yerba Mate and demonstrates their importance in the production of bioactive compounds. Further research on cultivation and processing can be explored to maximize production of bioactive compounds for use in foods, crops, pharmaceuticals, and supplements to support human, animal and plant health.

Vita

Kellie Parks Burris was born on September 5, 1978 in Morganton, NC to Tom and Gail Parks. She attended Burke County Public Schools and graduated with honors from Freedom High School in 1996. Kellie attended North Carolina State University and graduated *Cum Laude* with two Bachelors of Science Degrees in Animal Science and Poultry Science in May 2000 and *Magna Cum Laude* with a Bachelors of Science Degree in Food Science with a minor in nutrition in May 2001. In August 2002, Kellie attended graduate school at the University of Tennessee, and graduated with her Masters of Science degree in Food Science and Technology in December 2004. She obtained a second Masters of Science degree at the University of Tennesssee in Plant Sciences in December 2006. While finishing her second Masters of Science, she became a partner and the Research Scientist and Laboratory Manager for the start-up biotechnology company, MycoGenomix, LLC. In January 2009, Kellie began her Doctor of Philosophy in Food Science and Technology at the University of Tennessee. Her research focused on antimicrobial discovery from botanicals and pathogen detection. Kellie plans on pursuing a career in research in industry or academia.