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The Efficacy of Antibiotic Residue Screening Tests for the Detection of Natural Antimicrobials in Milk

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The Efficacy of Antibiotic Residue Screening Tests for the Detection of Natural
Antimicrobials in Milk.

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The Efficacy of Antibiotic Residue Screening Tests for the Detection of Natural
Antimicrobials in Milk.

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LIST OF ABBREVIATIONS

Introduction and Literature Review

DCT = Dry Cow Therapy
FDA = Food and Drug Administration
GRAS = Generally Recognized as Safe
h = hour(s)
IMM = intramammary
MBC = Minimum Bactericidal Concentration
MF = Milk Fat
MIC = Minimum Inhibitory Concentration
min = minute(s)
mo = month(s)
MP = Milk Protein
NCIMS = National Conference on Interstate Milk Shipments
NDRMMP = National Drug Residue Milk Monitoring Program
NOP = National Organic Program
SCC = Somatic Cell Count
USDA = United States Department of Agriculture
yr = year(s)

Materials and Methods, Results and Discussion

BDL = Below Detection Limit
d = day
DL = Detection Limit
PMO =Pasteurized Milk Ordinance
ppb = parts per billion

INTRODUCTION

Mastitis is the most widespread disease in dairy cattle affecting animal health and the disease results in serious financial losses to dairy farmers (Pol and Ruegg, 2007). Currently, intramammary **(IMM)** infusion of antibiotics to treat mastitis is the single greatest reason for the use of antibiotics in dairy cows (Pol and Ruegg, 2007).

In the United States, there are a limited number of antibiotics approved for IMM treatment of mastitis (Pol and Ruegg, 2007). β-lactams are the primary class of antibiotics that have been approved by the Food and Drug Administration **(FDA)** for the treatment of mastitis (Holstege et al., 2002). To ensure human food safety, the FDA is responsible for establishing milk withholding times, "safe" or "tolerance" levels for antibiotics in milk, and analyzing all bulk types of commingled milk for the presence of β-lactam and other drug residues. To detect violative concentrations of these residues in milk, the FDA uses qualitative, rapid antibiotic residue screening tests to monitor for β-lactams and other classes of antibiotics and drugs (FDAa, 1997).

Detecting violative levels of antimicrobial residues in commingled milk through the use of residue screening tests can help prevent contaminated milk from entering the human food supply. Testing at this level, however, does not protect the dairy producer from the loss of milk and financial penalties due to a contaminated bulk tank (Andrew, 2000). Although milk is not regulated at the individual cow basis, a study conducted by McEwen et al. (1991) determined that the use of antibiotic screening tests for milk from individual cows was associated with reduced risk of bulk milk residue incidence when used under field conditions.

Even though commingled milk is tested for antibiotic residues, some consumers remain concerned about the possibility for contaminants in milk (Hillerton et al., 1999). Concerns regarding the perceived or potential harmful effects of antibiotic residues in milk have, in part, driven the consumer demand for organically produced dairy products (McEwen et al., 1991), where the use of antibiotics in cows supplying milk for organic production is prohibited (Pol and Ruegg, 2007). Dairy products are only second (16%) to fruits and vegetables (40%) in organic food purchases (Organic Trade Association, 2007). The demand for organic milk is predicted to grow and potentially may result in a dramatic increase in the proportion of dairy farms transitioning to organic practices (Ruegg, 2009).

In the United States, organic milk can only come from cows that have never been treated with an antibiotic (Karreman, 2007) and there are no "natural products" that have been evaluated and approved by the FDA for IMM use. There are many natural remedies that are topical and pose little risk for contamination of milk or meat (Ruegg, 2009). A few of these "natural treatments" have the potential to be efficacious in treating mastitis by IMM infusion; however, research is lacking to support the efficacy of these compounds.

Natural antimicrobials such as caprylic acid, eugenol, *trans*-cinnamaldehyde, carvacrol, and thymol have been found to be inhibitory, *in vitro*, towards an array of pathogenic microorganisms and may be potential candidates for efficacious non-antibiotic treatments for mastitis (Ananda Baskaran et al., 2008, Nair et al., 2005). If so, then evaluation of possible residues would be an important part of the use of these compounds in commercial practice.

Screening tests may prove to be a valuable tool in evaluating the presence of "natural" antimicrobials in milk, even if there are no human health concerns with the residues of these compounds. Other concerns of "natural compounds" in milk may be the effect on the taste of milk or interfering with the culturing process for cheese and yogurt. Antibiotic residue screening tests may be useful to regulatory agencies, processing plants and producers for assurance of safety of milk produced by conventional and organic dairy farms, if the test can detect "natural antimicrobials." This study evaluated the efficacy of antibiotic residue screening tests for the detection of natural antimicrobials in milk.

LITERATURE REVIEW

Mastitis

Mastitis is considered the most prevalent and expensive disease of dairy cattle worldwide. Specifically, mastitis is an inflammatory reaction of the mammary gland usually in response to pathogenic microorganisms. An infection occurs when microorganisms breach the teat canal and multiply within the mammary gland thus eliciting the inflammatory response. Infections may be clinical or subclinical, depending on the extent of inflammation (Schrick et al., 2001).

Clinical mastitis is distinguished by visible abnormalities in the appearance of the udder or milk. The infected quarter may be swollen, tender, and warm. Decreased milk production and alterations in the milk such as the presence of clots, flakes, and discoloration may also be apparent in clinical cases (Philpot and Nickerson, 1991).

Subclinical mastitis is more subtle and is not detected by visual observation and can only be identified from a culture of milk containing the mastitis causing pathogen. A

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possible sign of subclinical mastitis is that the cow may have an increased somatic cell count **(SCC)** due to the infection. The subclinical form is significantly more common than the clinical form (Philpot and Nickerson, 1991). Subclinical mastitis can also cause a chronic decrease in milk production that results in several thousand pounds of milk lost per year **(yr)**, if left untreated (Philpot and Nickerson, 1991).

Depending on the causative agent, mastitis can be broadly classified into contagious mastitis and environmental mastitis. Contagious microorganisms reside in the mammary gland and can spread from one cow to another during the milking process. Environmental microorganisms, however, are found in the surroundings in which a cow is housed and can infect the mammary gland usually at times other than milking. *Staphylococcus aureus, Streptococcus agalactiae,* and *Mycoplasma bovis* are the primary pathogens that cause contagious mastitis. The primary pathogens that cause environmental mastitis are *Klebsiella pneumoniae*, *Escherichia coli, Streptococcus dysgalactiae,* and *Streptococcus uberis*. Although, contagious pathogens are responsible for the majority of mastitis cases, environmental pathogens are becoming a principal concern as well (Pyörälä, 2002; Erskine et al., 2003).

Mastitis is the single most expensive disease in the dairy industry. Mastitis can lead to severe economic losses due to reduced milk production, discarded milk, culling, delayed genetic progress, veterinary services, and medication (Philpot and Nickerson, 1991). Worldwide losses are estimated to be $35 billion (Wellenberg et al., 2002). The National Mastitis Council of the United States estimated the annual dollar loss to the dairy industry due to mastitis to be approximately $2 billion. In another perspective, this amounts to approximately $180 per cow per yr (Harmon, 1996). Since mastitis is both an

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animal health concern and economic concern, it is in the producer's best interest to act swiftly to prevent and combat the disease.

Treatments for Mastitis

The most common method available for treating mastitis is IMM infusion of antibiotics. In the United States., there are a limited number of antimicrobial drugs that can be marketed for IMM treatment of mastitis. The current FDA approved antibiotic classes include β-lactams, macrolides (erythromycin), coumarines (novobiocin), and lincosamides (pirlimycin) (Pol and Ruegg, 2007). Of these four classes, β-lactams are the most common antibiotics used for IMM treatment of mastitis on conventional dairy farms (Pol and Ruegg, 2007). The six main antimicrobials that make up the β-lactam class are ampicillin, amoxicillin, ceftiofur, cephapirin, cloxacillin, and penicillin G (Holstege et al., 2002).

The use of IMM antibiotics at dry off is common in United States dairy facilities. Dry cow therapy **(DCT)** is usually administered as a treatment for existing subclinical cases of mastitis and as a means of prevention during the non-lactating period (Pol and Ruegg, 2007). There are a number of advantages for DCT including; the cure rate is generally greater than when treated during lactation, higher concentrations of long-lasting antibiotics may be used safely, the incidence of new infections during the dry period is reduced, and salable milk is not contaminated with drug residues (Philpot and Nickerson, 1991). A national survey of dairy herds (USDA/APHIS/VS/CEAH, 2005) reported that more than 75% of producers administered IMM DCT in all cows within a herd. The sample survey reported that cephapirin was the most commonly used IMM antibiotic (42%), followed by penicillin (32%), and cloxacillin (13%) (Pol and Ruegg, 2007).

Even though the most common mastitis treatment is IMM infusion of antibiotics, this method has some limitations. The cure rates are generally low against many mastitis causing agents. The cure rates of *Staphylococcus aureus* infections, for example, typically vary between 20 and 78% (Dingwell et al, 2003). Another concern is the overuse of antibiotics to treat bacterial diseases in cattle which has been suggested as a catalyst for the emergence of antibiotic-resistant strains of bacteria (White, 1999). Additionally, antibiotics used to treat mastitis have been implicated to be the most common source of harmful drug residues in milk (Erskine, 1996). Approximately 90% of inhibitory residues detected in milk over a five yr span in Michigan originated from antibiotic treatment for mastitis (Erskine et al., 2003). Due to these concerns, commingled milk must be monitored closely to avoid violative levels of antibiotic residues.

FDA regulations for the presence of antibiotics in milk

The presence of antibiotic residues in milk is a primary concern for the dairy industry. These residues have been linked to allergic reactions (in sensitive individuals), emergence of resistant bacterial strains, and the impairment of bacterial fermentation processes (Sierra et al., 2009).

The FDA is responsible for regulating commingled milk. In 1991, the FDA, with the support of the National Conference on Interstate Milk Shipments **(NCIMS),** initiated the National Drug Residue Milk Monitoring Program **(NDRMMP)** (FDAa, 1997).

The NDRMMP is designed to provide an estimate of the rate of animal drug residues that may be present in milk. Under the NDRMMP, all tankers of milk in the United States must be screened for residues of β-lactam antibiotic drugs. The only way

to comply with this requirement, without delaying the delivery of milk, is through the use of rapid, qualitative antibiotic residue screening tests (Kijak, 2004).

The FDA developed a validation program for these screening tests for regulatory use. To be considered for regulatory uses, a screening test must be able to detect four of the six β-lactam antibiotic drugs, at or below the legal tolerance level or "safe level" (Kijak, 2004). Milk containing antibiotic residues at or above FDA established tolerance or "safe levels" are considered violative. Residues in concentrations below the established levels are not considered to be of public health or regulatory significance (FDAa, 1997). The program has been successful as evidenced by a marked decrease in violative levels of milk from 0.104% in 1996 to 0.026% in 2009 (FDAb, 1997, FDAb, 2010).

There are strict financial penalties for producers if antibiotic residues are detected at or above the tolerance or "safe" levels in their milk. The producer's Grade "A" permit will be suspended and not reinstated until their farm is investigated and the cause of the contamination is identified and corrected. The producer must also discard all their milk for a withholding period, ranging from one to three days. In addition, the producer must purchase any other farms' milk that was mixed on the tanker truck. The producer is also responsible for the cost of transportation and disposal fees (FDAa, 1997).

Antibiotic Residue Screening Tests

Antibiotic residues must be monitored to prevent contaminated milk from entering the human food supply. The best way to monitor commingled milk is through the use of residue screening tests. These tests are rapid, qualitative, and can detect a broad range of antibiotic residues (Navrátilová, 2008).

Dairy facilities and government agencies use residue screening tests to comply with the NCIMS requirement that all tankers of milk in the United States must be screened for β-lactam antibiotic residues (Kijak, 2004).

There are many different types of FDA approved antibiotic residue screening tests that may be used to detect the presence of β-lactam drug residues. The most commonly used screening tests include microbial growth inhibition assays, microbial receptor assays, receptor binding assays, immunologic assays, and enzymatic assays (Kang et al., 2005).

Microbial growth inhibition assays make use of a standard culture of the tested microorganism (i.e. *Bacillus stearothermophilus)* in a solid or liquid medium. The milk sample is added to the agar surface and allowed to incubate. Over the duration of incubation, the sample diffuses into the medium, if the sample contains inhibitor agents such as β-lactam drug residues, reduction or complete inhibition occurs of the tested microorganism growth (Navrátilová, 2008). Microbial growth inhibition assays differ in the type of the testing organism, indicator, incubation period and temperature, and detection levels of the agents analyzed. One of the most common microbial inhibition tests is the Delvotest[®] which is used primarily as an "on-farm" screening method for bulk tank and individual cow's milk (Andrew, 1997).

The Delvotest[®] is qualitative, sensitive, and detects a broad range of antibiotics based on the rapid growth and acid production of the test organism, *Bacillus stearothermophilus* var. *calidolactis* (Kang et al., 2005). The test kit consists of agar ampoules containing spores of the test organism and nutrient. The spores of the bacterium will germinate and produce carbonic acid after the addition of milk and

subsequent incubation. The production of carbonic acid causes the bromocresol purple indicator in the ampoule to change color from purple to yellow. An ampoule that has turned yellow after incubation is indicative of a negative sample. When antimicrobials are present in the milk sample, the growth of the bacteria is inhibited and the color of the agar remains purple, signifying a positive result (LeBreton et al., 2006). The advantages of this type of assay include a wide detection spectrum, ease of use, and ability to screen for a large number of samples (Navrátilová, 2008).

Receptor binding assays are also a common class of antibiotic residue screening tests. This type of assay involves a receptor protein conjugated to an enzyme. The conjugate will bind to free β-lactam antibiotics that may be present in the milk sample (Navratilova, 2008). One of the most common receptor binding assays is the IDEXX β-Lactam SNAP test.

The β-lactam SNAP test is an enzyme-linked, receptor binding assay in which β-lactams are captured by a binding protein on a solid support adsorbent matrix. The enzyme conjugate binds with the β-lactams present in the milk and the mixture is then transferred to the sample well of the device. The sample travels on a filter paper strip until it passes to the test spot. The test spot is coated with β-lactam antibiotic which causes free receptors to become bound to the spot. The substrate is then released and reacts with the enzyme bound to the captured receptor protein causing a color to develop at the test spot. If the color of the test spot is weaker than that of the control spot, the result is indicative of a positive. The main advantages of this type of assay are its ease of use and rapid testing time, which is approximately ten minutes (Navratilova, 2008) and these specific, rapid; tests are primarily used by processors and regulator personnel.

Another common type of screening test is the enzymatic assay. The main type of enzymatic assay is the Penzyme screening test. The Penzyme test is a qualitative, enzymatic colorimetric assay for quick determination of β-lactam residues present in milk. The test principle is based on establishing the level of inactivation of the (DD-carboxypeptidase) enzyme by β-lactam antibiotics. The residues will bind specifically with the enzyme and inactivate it. The milk sample is added to an ampoule that contains the enzyme *Streptomyces* DD-carboxypeptidase. During incubation, any β-lactam residues present in the sample will create a stable complex with the enzyme. The degree of inactivation depends on the amount of antibiotics present in the milk sample. After a reagent pellet is added, incubation takes place. If the sample does not contain β-lactam residues, the enzyme is hydrolyzed eventually giving rise to two end products, pyruvic acid and hydrogen peroxide. Hydrogen peroxide is used to oxidize the organic redox indicator. If the sample is negative, the indicator will change into a pink-orange color. If a yellow or yellow-orange color is observed, the sample is suspected of containing an antibiotic residue (Navratilova, 2008). The two main advantages of this assay are the ease of the use and rapid testing time. Although residue screening tests can detect a wide range of antibiotics and are simple to use, they are not without their short-comings.

Concerns associated with these qualitative residue screening tests are the variable rates of sensitivity and specificity across tests and antibiotics. Sensitivity refers to the likelihood of correct identification of a positive (milk containing a violative concentration of an antibiotic). Specificity refers to the likelihood of correct identification of a true negative; milk without violative concentrations of an antibiotic (Van Eenennaam et al., 1993). Van Eenennaam et al. (1993) evaluated the specificity and sensitivity of the

Delvotest® and the CITE SNAP test with milk from cows with mild to moderate clinical

mastitis. Sensitivity values were determined from quarter milk samples of the antibiotic

therapy groups after the antibiotic was administered, because these were the only samples

expected to contain detectable levels of antibiotics. Specificity values were determined

from the milk samples of untreated cows because the samples were expected to be free of

antibiotic residues. The Delvotest® was determined to have a high sensitivity (97%) but a

low specificity (38%) (Van Eenennaam et al., 1993). The CITE SNAP also had a high

sensitivity (85%) but a low specificity (30%). A possible explanation for the low

specificity of the Delvotest® and CITE SNAP is that SCC increase in concentration in

milk from infected quarters and have been known to raise the incidences of false positive

readings. The rate of false positive outcomes is much greater when milk from a cow with

clinical mastitis is tested compared to evaluating milk from a normal quarter or quarter

with sub-clinical mastitis (Andrew, 2000).

Andrew (2000) evaluated the specificity rates of four different residue screening

tests including the Delvotest®, the CITE SNAP, and the Penzyme screening test. The

Delvotest® had a high specificity rate of 1.0 when testing milk from Holstein cows and a

rate of 0.98 when testing milk from Jersey cows. The CITE SNAP also had a high

specificity rate of 1.0 when testing milk from Holstein cows and a rate of 0.93 when

testing milk from Jersey cows. The Penzyme test had a low specificity rate of 0.77 when

testing milk from Holstein cows and an even lower rate of 0.53 when testing milk from

Jersey cows. Andrew, (2000) concluded the breed difference was due to the difference in

milk fat **(MF)** and milk protein **(MP)** percentage between the two breeds. Jerseys

typically have greater MF and MP percentages in comparison to Holsteins, and

increasing MF percentage was associated with an increase in the probability of false-positive outcomes for the CITE SNAP test (Andrew, 2000). Increased MP and increased SCC were associated with an increase in false-positive outcomes for the Penzyme test. Due to the fact that the Penzyme test is an enzymatic assay, the proteins from the milk samples may have interfered with the test results (Andrew, 2000). Based on the results of this study and previous studies, the CITE SNAP and Delvotest® antibiotic residue screening tests can be used to evaluate the antibiotic residue levels of milk from Jersey cows which may have elevated MF and MP production levels (Andrew, 2000).

Organic Agriculture

Organic milk production has become one of the fastest growing sectors of organic agriculture. In 2007, the Organic Trade Association reported that sales of organic foods increased by 18% to represent $20 billion in consumer sales. In addition, dairy products (16%) are only second to fruits and vegetables (40%) as a percentage of overall organic food purchases (Organic Trade Association, 2007). The United States Department of Agriculture **(USDA)** has estimated that organic milk increased from 2% of United States fluid milk purchases in 2006 to 3% in 2008 (USDA, 2008).

Consumer demand for organic dairy products has led to a dramatic increase in the proportion of dairy farms switching to organic practices (Ruegg, 2009). Between 2000 and 2005, the number of certified organic dairy cows in the United States increased from 38,000 to more than 86,000 (USDA, 2006). A majority of these cows were housed on small, conventional dairy facilities that had transitioned to organic production to increase farm profitability (USDA, 2008). In 2005, Wisconsin housed the highest percentage (19%) of organic dairy cows in the United States (Ruegg, 2009).

The increased consumer demand for organic foods is, in part, driven by perceived concerns about the safety of foods produced on conventional facilities. Consumers are concerned about potential health risks associated with the use of pesticides and antibiotics in food production (Ruegg, 2009). Organic dairy products are perceived to be a safer choice by the public because organic dairy cattle are raised on organic feeds (grown without the use of pesticides), antibiotics and growth hormones are prohibited, and husbandry practices are intended to limit stress and promote the health of the dairy herd (Ruegg, 2009).

The process of organic accreditation is becoming increasingly more regulated. The USDA National Organic Program **(NOP)** has defined the standards for organic production and handling within the United States (USDA, 2008). These standards address the methods, practices, and substances used in producing and handling crops, livestock, and processed agricultural products. Under the USDA guidelines, dairy products must be from animals that have been under continuous organic management for a minimum of one yr, except during the transition period when entire dairy herds are being converted to organic practices. For the first nine months **(mo)** of the yr of transition, the producer is allowed to feed the herd a minimum of 80% organic feed (Ruegg, 2009). Once the transition period is completed, the herd must be fed organic feeds and housed in conditions that provide exercise and freedom of movement to minimize stress to the animal (USDA, 2008).

One of the most important regulations under the USDA NOP is that most conventional therapeutic compounds are prohibited for use in dairy cattle on organic facilities. These compounds include but are not limited to β-lactam antibiotics,

tetracycline, sulfa drugs, steroids, growth and reproduction hormones, and recombinant bovine somatotropin (rbST / rBGH / bovine growth hormone). Under current guidelines, if a prohibited agent is used, the animal will be ineligible for organic milk production forever (Karreman, 2007).

USDA NOP regulations require producers to provide appropriate medical treatment to ill cows, but animals that receive treatment from a prohibited source (i.e. antibiotic, hormone, steroid, etc.) are immediately and permanently disqualified from organic production. All appropriate treatments and medications must be used to restore the health of the animal when methods acceptable to organic standards fail to alleviate the condition (USDA, 2008). Unfortunately, there are no FDA-approved antimicrobial compounds on the USDA-approved list of allowed organic treatments. In addition, the FDA guidelines do not allow the use of unapproved substances for IMM therapy, regardless of whether or not the compound is a natural compound such as a botanical, homeopathic agent, or food supplement, for the treatment of food-producing animals even under the supervision of a veterinarian.

Natural Treatments

a. Alternative products for the treatment of mastitis

The treatment of mastitis accounts for the majority of antibiotic usage on conventional facilities. The most common treatment method for mastitis is IMM infusion of antibiotics. This method, however, is not available to organic dairy producers (Ruegg, 2009). Organic dairy practices must rely on natural products to treat for mastitis.

In a survey conducted by Pol and Ruegg (2007), Wisconsin farmers reported the rate of use of natural products for treating mastitis; whey-based products, garlic tincture,

and aloe were the most commonly used products with a reported usage rate of 45%, 35% and 30% respectively. The use of aloe is common because it is believed to drain the infection and soften the udder when it is infused into the quarter. There is no FDA approval, however, for the IMM use of this and other natural compounds (Ruegg, 2009). Wisconsin farms surveyed also reported the usage rates of Vitamin C, Aspirin, and vegetable oils at 25%, 20%, and 20% respectively (Pol and Ruegg, 2007). Topical herbal ointments containing ginger, peppermint, or lemon extracts are also commonly used and can be applied to reduce inflammation, although studies have not been done to determine the efficacy of these products (Karreman, 2007). There have been very few peer-reviewed studies that have dealt with the clinical efficacy of natural products for treating mastitis (Ruegg, 2009).

b. Natural antimicrobials

Several natural lipids have been shown to be effective against microbial growth. Studies involving fatty acids and their monoglycerides demonstrated that these compounds could inhibit a variety of pathogenic organisms (Kabara, 1978; Isaacs et al., 1995; Petschow et al., 1996). Nair et al. (2005) evaluated the antimicrobial activity of caprylic acid and its monoglyceride, monocaprylin, against mastitis causing pathogens. Both caprylic acid and monocaprylin were determined to be bactericidal against the major bovine mastitis pathogens in milk. In this study, *Staphylococcus aureus* in milk was reduced from 6.0 log cfu/mL to 1.0 log cfu /mL after six hours **(h)** of incubation (Nair et al., 2005).

Plant-derived natural oils have also been studied for their antimicrobial activity. *Trans*-cinnamaldehyde, eugenol, carvacrol, and thymol are a group of generally

recognized as safe **(GRAS)** natural antimicrobials that are traditionally used to preserve

food and enhance flavor. A study conducted by Ananda Baskaran et al. (2009)

determined the efficacy of these natural antimicrobials against mastitis causing pathogens

(*in vitro*). The researchers determined the minimum bactericidal concentration **(MBC)**

and minimum inhibitory concentration **(MIC)** of each of the natural compounds against

mastitis causing pathogens and determined the efficacy of each of the compounds. All

four compounds were effective against each of the five mastitis pathogens tested but

trans-cinnamaldehyde had the greatest bactericidal activity. *Trans*-cinnamaldehyde at

the established MBC (0.45%) reduced pathogen populations from 6.0 log cfu/mL to

undetectable levels after 24 h for each of the five pathogens tested (Ananda Baskaran et

al., 2009).

In addition, a commercial herbal remedy, phyto-mast[®], is marketed as a potential

treatment for mastitis in cows in organic dairy production. This product is composed of

several organic compounds including *Angelica sinensis*, *Glycyrrhiza uralensis*,

Gaultheria procumbens, and *Thymus vulgaris* (McPhee et al., 2009). There have been

recent studies conducted to evaluate the efficacy of phyto-mast[®] as a viable treatment for

mastitis. Mullen et al. (2010), compared the efficacy of three different IMM treatments

for mastitis; phyto-mast[®], antibiotic treatment, and no treatment, as a DCT. The

researchers found that conventional antibiotic treatment had the highest cure rate and

lowest new infection rate. Cows treated with phyto-mast[®] however, had fewer new

infections compared to cows receiving no treatment. Even though phyto-mast[®] was less

effective than antibiotic treatment, the researchers concluded that phyto-mast[®] has the

potential to be a useful dry cow treatment on organic dairy farms (Mullen et al., 2010). A

study conducted by Pinedo et al. (2009), evaluated the efficacy of phyto-mast® in treating clinical mastitis. The researchers reported that cows treated with phyto-mast® had a faster recovery rate and a higher bacteriological cure rate compared to untreated cows (Pinedo et al., 2009). There have been no FDA evaluation of this product therefore; the efficacy of this compound has not been established.

Nisin is a natural compound that is being studied for its efficacy in treating mastitis. Nisin is an antimicrobial polypeptide that is produced by *Lactococcus lactis.* It is a GRAS compound and it has been formulated into products used for teat-dipping; however, there are few reports on the use of nisin as an IMM mastitis therapy (Sears et al., 1992). Cao et al. (2007) evaluated the efficacy of nisin compared to gentamicin, an antibiotic for the treatment of clinical mastitis in lactating dairy cows. The researchers found that there was no difference between the two antimicrobials in regards to bacteriological cure rates and clinical cure rates. Nisin however, was significantly more effective at eliminating *Staphylococcus aureus* infections compared to gentamicin (Cao et al., 2007).

In a study done by Zhou et al. (2007), thymol and carvacrol were assessed alone and in combination against *Salmonella typhimurium* to determine the antimicrobial effects of the compounds. The combination of the two antimicrobials was more effective at reducing the populations of the microorganism compared with milk spiked with thymol and carvacrol alone (Zhou et al., 2007). Johny et al. (2010) reported similar findings. The combination of *trans*-cinnamaldehyde, eugenol, thymol, carvacrol, and *b*-resorcylic acid was more effective than the individual compounds at rendering *Salmonella typhimurium* susceptible to antibiotics. A study done by Brandt et al. (2010)

evaluated nisin alone and in combination with acidic calcium sulfate against *Listeria monocytogenes* to determine the ability of the compounds to inhibit the growth of the pathogen. The combination of nisin with acidic calcium sulfate was more effective than the individual compounds at reducing the growth of *Listeria monocytogenes* (Brandt et al., 2010). The results of these studies indicate that these antimicrobials are effective individually and the antimicrobial effects may be enhanced when the compounds are administered as a combination (Johny et al., 2010). The results from the above studies indicate that natural antimicrobials may have the potential to be used alone or in combination, as an alternative to antibiotics for the treatment of mastitis.

Pasteurization

The vast majority of milk is consumed after pasteurization. Pasteurization is the process of heat treating raw milk to inactivate or kill potentially harmful pathogens (Smith, 1981). There have been few studies that have evaluated the effect of heat on the antimicrobial activity of antibiotics. Zorraquino et al. (2008) tested the effect of varying heat treatments on milk spiked with different concentrations of β-lactam antibiotics. The results of the study showed that heating milk at 40°C for 10 minutes **(min)** did not cause heat inactivation for any of the antibiotics whereas milk heated at 83°C for 10 min resulted in significant losses in activity for penicillin G, cephalexin, and cefuroxime. Milk heated at 60°C for 30 min, however, resulted in a minimal loss in antimicrobial activity for the antibiotics analyzed (Zorraquino et al., 2008). The heat treatment at 60°C for 30 min was near the level of the pasteurization. There are very specific requirements for pasteurization, milk must be heated at 63°C for 30 min, then rapidly cooled in an ice

bath (Smith, 1981). Natural antimicrobials should also be evaluated in pasteurized milk to determine if heat causes a loss in their antimicrobial activity.

In summary, with the interest of natural compounds that could potentially be used as treatments for mastitis, there comes the threat of antimicrobial residues. Milk must be monitored to prevent contaminated milk from entering the human food supply. Antibiotic residue screening tests may be able to detect the presence of these natural antimicrobials in milk. Naturally derived compounds could possibly interfere with the production of cultured products, such as cheese and yogurt. If antibiotic residue screening tests are able to detect the presence of antimicrobials then this problem could be avoided. Screening tests can give producers a means of ensuring the quality of their products and safety of their milk. This study evaluated the efficacy of antibiotic residue screening tests for the detection of natural antimicrobials in milk.

HYPOTHESES

The hypotheses of this study are fourfold:

1) Qualitative antibiotic residue screening tests, particularly microbial inhibition tests can detect natural antimicrobials in milk.

2) For natural antimicrobials that have established MIC values, it is expected that these screening tests can detect the natural compounds at their MIC and concentrations below the MIC (detection limits).

3) Pasteurization does not affect the ability of the residue screening tests to detect the natural compounds.

4) The combination of natural antimicrobials increases the risk for residues in milk.

OBJECTIVES

The objectives of this experiment are to determine if the Delvotest® SP-NT and the IDEXX β –Lactam SNAP test can detect natural antimicrobial compounds in milk, determine the sensitivity of these antibiotic residue screening tests, and determine the detection limits for each of these compounds, as well as the effect of pasteurization on the antimicrobial activity of the compounds and the effect of the combination of compounds on detection by antibiotic residue screening tests.

MATERIALS AND METHODS

Animal Selection and Milk Collection

Quarter composite milk was collected from 59 Holstein and 37 Jersey cows from the University of Connecticut Kellogg Dairy Center during the afternoon milking. Milk was collected from cows with visually normal milk and mammary gland. Cows had not been treated with an antibiotic for at least 30 days (**d**) prior to collection. Milk from Holstein and Jersey cows was sampled to provide a wide range in MF and MP contents.

The teats were prepared for milking based on the University of Connecticut Kellogg Dairy Center's general operating procedures. Briefly, teats were pre-dipped with an iodine-based product and dried with individual cloth towels. Three streams of foremilk from each quarter were discarded before sample collection. Fifty mL of quarter composite milk was collected from each cow.

Milk Fat Content, Milk Protein Content, and Somatic Cell Count Analyses

A subsample of five mL of milk, taken from a composite milk sample, was analyzed for MF and MP contents by infrared spectroscopy (DAIRY ONE, DHI Milk Testing Laboratory, Ithaca, NY). Another subsample of five mL of milk, taken from the 50 mL quarter composite milk sample was analyzed for SCC at the University of Connecticut Veterinary Medical Diagnostic Laboratory. The SCC of each milk sample was determined using the DeLaval cell counter DCC (DeLaval Inc., Kansas City, MO). Five additional milk samples with SCC greater than 200×10^3 SCC/mL were analyzed for each compound to determine if elevated SCC interfered with the accuracy of the screening tests.

Caprylic Acid, Plant-Derived Antimicrobials, and Nisin

Caprylic acid, the plant-derived essential oils; *trans*-cinnamaldehyde, eugenol, carvacrol, and thymol, (Sigma-Aldrich Chemical Company, St. Louis, MO), phyto-mast[®] (Penn Dutch Cow Care, Narvon, PA), and nisin (ImmuCell Corporation, Portland, Maine) were evaluated for detection in milk using antibiotic residue screening tests. The compounds, reference numbers and MIC, if known, are presented in Table 1.

Caprylic acid is a medium-chain fatty acid naturally present in milk at low concentrations (Jensen et al., 1990; Sprong et al., 2001; Jensen, 2002). *Trans*-cinnamaldehyde is the primary component of cinnamon oil (Ananda Baskaran et al., 2009). Eugenol is an active agent in clove oil (Ali et al., 2005), and carvacrol and thymol are ingredients in oregano oil (Bendahou et al., 2008).

Phyto-mast[®], unlike the other antimicrobials, is a commercial product that contains a mixture of plant-derived essential oils that include *Angelica sinensis*, *Glycyrrhiza uralensis*, *Gaultheria procumbens,* and *Thymus vulgaris. Angelica sinensis* is a flavoring agent for gin, *Glycyrrhiza uralensis* is a component of licorice, *Gaultheria procumbens* is a source of wintergreen flavoring, and *Thymus vulgaris* is a thyme flavoring additive (McPhee et al., 2009). Nisin is an antimicrobial polypeptide that is produced by *Lactococcus lactis* (Sears et al., 1992).

Determination of the MIC for Phyto-mast[®]

The method described by Andrews (2001) was used to determine the MIC of the phyto-mast[®]. Milk containing phyto-mast[®] in the range of 0 to 67% (vol/vol) was inoculated with each of the mastitis pathogens; *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysagalactiae.* Following incubation, the samples were plated

onto tryptic soy agar plates. The plates were then analyzed to determine the minimum

concentration of phyto-mast® that inhibited the growth of each pathogen (Ananada

Baskaran et al, 2009).

Preparation of spiked milk samples

For each compound, 30 replicates using individual milk samples from 15 Holstein

and 15 Jersey cows, were evaluated for determination of sensitivity by the antibiotic

residue screening tests at the MIC, except for phyto-mast®. Two lower concentrations

were identified for each compound that represented the lowest concentration that would

result in a test sensitivity rate of greater than 0.90 (the detection limit) and the greatest

concentration of the compound where the sensitivity rate was less than 0.03 (below

detection limit). These two concentrations were determined by screening each compound

in small increments over a wide range of concentrations. Within each compound, the

same milk samples were used to determine sensitivity for the three concentrations.

Combination of antimicrobials

Eugenol was combined with either *trans*-cinnamaldehyde or thymol at their

respective concentrations below the detection limit and evaluated in milk by the residue

screening tests. For each combination, three replicates were tested using individual milk

samples from lactating dairy cows.

Pasteurization of spiked milk samples

Each compound was evaluated in triplicate, at the MIC and detection limit, to

determine if pasteurization had an effect on the antimicrobial activity of the compounds.

The spiked milk samples from individual cows were heated in a water bath at 63° C for

30 min and then immediately cooled in an ice bath for 60 min as in accordance with the

pasteurized milk ordinance (**PMO**) standards for batch pasteurization (FDA, 2009).

Determination of sensitivities and detection limit of compounds

The spiked milk samples were analyzed for the presence of inhibitors by two

commercially available, antibiotic residue screening tests, the Delvotest® SP –NT

(Nelson-Jameson Inc., Marshfield, WI) and the SNAP β-lactam test (IDEXX

Laboratories Inc., Westbrook, ME). The Delvotest® SP –NT is a microbial growth

inhibition test that detects β-lactam and sulfur antibiotics and the SNAP β-lactam test is

an antibiotic-antigen capture test.

For each day of analysis, standard positive (Nelson-Jameson Inc., Marshfield, WI,

Delvotest® Penicillin G Standard Control, 5 ppb) and negative control samples (Nelson-

Jameson Inc., Marshfield, WI, Delvotest® Negative Control, 5 ppb) were analyzed along

with the milk samples for both residue screening tests to verify the accuracy of the tests.

Statistical Analyses

A sensitivity rate (the rate of truly positive samples that were found to be positive

by the Delvotest® SP-NT) and a 95% confidence interval were calculated for each

compound at each concentration (Van Eenennaam et al., 1993). A sensitivity rate of 1.0

indicated that all samples that contained a compound were found to be positive by the

Delvotest® SP-NT. SCC were converted to somatic cell scores and the effect of SCC,

MF, and MP on the sensitivity rates of the Delvotest® SP-NT was evaluated using PROC

LOGISTIC (SAS Inst, Inc., Cary, NC).

RESULTS AND DISCUSSION

Over a 14 mo period, 185 quarter composite milk samples were collected from 59 Holstein and 37 Jersey cows. Throughout the course of the study, several cows were sampled more than once at different stages of lactation. Mean MF and mean MP concentrations were typical of breed averages (Wiggans, 2006) and were greater for milk from Jersey cows than milk from Holstein cows, as expected (Table 2). The SCC ranged from 3×10^3 cells/mL to 841×10^3 cells/mL across both breeds. There were five additional milk samples collected from three Jersey and two Holstein cows, with a geometric SCC mean of 552×10^3 cells/mL and a range from 200×10^3 cells/mL to 5965×10^3 cells/mL. MP and MF contents, and SCC did not affect the outcome of the Delvotest® SP-NT and all results are summarized across breeds.

Phyto-mast® did not inhibit the growth of the mastitis pathogens and; therefore, the MIC could not be determined.   In a study by Pinedo et al. (2009), phyto-mast® was compared to no treatment for treating cows with clinical mastitis. The study found that there were no differences in cure rates between the two treatments but, there was a tendency for a faster recovery rate for cows treated with phyto-mast® compared to no treatment   A study conducted by Mullen et al. (2010) reported similar results in relation to cure rates between phyto-mast® and no treatment. Phyto-mast® had the same cure rates as no treatment for bacteria present in the quarters of dry cows; however, in contrast, phyto-mast® tended to be more effective at preventing new infections than no treatment (Mullen et al., 2010). Previous research and the present study both indicate that the product may not be effective against mastitis pathogens and has not been evaluated by the FDA.

The IDEXX SNAP β-lactam test did not detect any of the antimicrobials investigated in this study. The SNAP test is specific to the basic common structure of β-lactam antibiotics. The compounds tested do not have a similar ring structure and; therefore, it was not expected that the SNAP test would detect the compounds evaluated in this study.

All positive and negative control milk samples were identified correctly by the Delvotest® SP-NT. The Delvotest® SP-NT detected caprylic acid and the plant-derived antimicrobials at their MIC values with sensitivities of 1.0 (Table 3). The established detection limits **(DL)**, were lower than the MIC for these compounds, with the exception of *trans*-cinnamaldehyde. The MIC and DL were the same concentration for *trans*-cinnamaldehyde. The sensitivity rates of the Delvotest® SP-NT for the DL ranged from 0.93 to 1.0 for the compounds evaluated. The concentration, at which the antimicrobials could no longer be detected by the Delvotest® SP-NT, below the detection limit **(BDL)**, differed for each compound, and were below the MIC and DL for all of the compounds (Table 3). These results demonstrate that the bacteria, *Bacillus stearothermophilus,* of the Delvotest® SP-NT, was more sensitive to caprylic acid and the plant-derived natural antimicrobials than the sensitivities of the mastitis pathogens.

The sensitivity of the Delvotest® SP-NT for nisin was 1.0 at the MIC and the DL (Table 4). The DL of nisin, by the Delvotest® SP-NT, was 400 fold less than that of that of the MIC. The MIC of nisin is markedly lower than for the other compounds evaluated in this study (Table 1), but similar to the MIC for several conventional antibiotics; such as ampicillin and amoxicillin (Tenhagen et al., 2006). The residue screening tests are designed to detect antibiotics below their MIC and tolerance and/or safe levels to protect

human food safety (FDAa, 2010); therefore, it is expected that all compounds would be detected by the screening test below their respective MIC values.

Although the MIC for several mastitis pathogens could not be established for phyto-mast® in this study, the Delvotest® SP-NT detected phyto-mast® at a concentration of 12.5% in milk (Table 4). The BDL for phyto-mast® was established to be 6.25% in milk. These concentrations for the DL and BDL are much greater for phyto-mast® compared to the other compounds evaluated in this study and conventional antibiotics (Thornsberry et al., 1996). Phyto-mast® is a suspension of a mixture of compounds and the specific concentrations of each compound in the mixture have not been published. These results indicate that the test organism in the microbial inhibition test was more sensitive to the compounds in phyto-mast® than for *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysagalactiae* when phyto-mast® was at a concentration of up to 67% in milk. Although, the phyto-mast® mixture did not inhibit mastitis pathogen growth, it can be detected by the Delvotest® SP-NT when present at high concentrations in milk.

There was variability among the compounds in their sensitivities to the screening test organism. Nisin was the most sensitive compound with a DL 99.8% less than its MIC (Table 5). *Trans*-cinnamaldehyde was the most sensitive of the plant-derived essential oils. The MIC of *trans*-cinnamaldehyde was less than the DLs of the other compounds evaluated, with the exception of nisin. The sensitivities of the screening test organism for caprylic acid, eugenol, and carvacrol were similar with DLs of 60%, 66.9%, and 70% less than their MIC, respectively. The DL for thymol demonstrated that the

screening test had a lower degree of sensitivity compared to that of the other compounds tested in this study.

The efficacy of the Delvotest® SP-NT to detect the plant-derived essential oils may be due to the high degree of hydrophobicity which enable them to target the lipid-containing cell membrane of the test bacteria, *Bacillus stearothermophilus*, a Gram-positive bacterium, in the screening test (Knobloch et al., 1986, Sikkema et al., 1994). These compounds may have disrupted the membrane of the *Bacillus stearothermophilus,* present in the agar, inhibiting its growth (Cox et al., 2000; Carson et al., 2002; Ultee et al., 2002). The antimicrobial action of caprylic acid, the medium-chain fatty acid, may be via its ability to diffuse across the cell membranes of bacteria and subsequent inhibition of growth of the bacteria (Nair et al., 2005). Several studies indicate that Gram-negative bacteria are less sensitive to the antimicrobial effects of fatty acids than Gram-positive bacteria (Kabara, 1978; Monk et al., 1996).

Nisin has been shown to have high antibacterial activity and because of this it is commonly used as a food preservative; especially for canned foods and dairy products (Cao et al, 2007). Gram-positive bacteria are especially sensitive to the bactericidal activity of nisin (Deegan et al, 2005). This may be a possible explanation for why the screening test was highly sensitive to nisin.

The pasteurization of milk did not have an effect on the outcome of the Delvotest® SP-NT, indicating that the antimicrobial activity of the compounds were not affected by heat treatment. This result is similar to the result reported by the Zorraquino et al. (2008) in that milk spiked with β-lactam antibiotics, heated at 60°C for 30 min, caused minimal to no loss in the antimicrobial activity of the antibiotics. Also, the

Delvotest® SP-NT detected the combination of eugenol and *trans*-cinnamaldehyde and eugenol and thymol at their respective concentrations below their DL, thus demonstrating the additive antimicrobial activity of eugenol combined with either *trans*-cinnamaldehyde or thymol. When these antimicrobials were analyzed alone below their respective DL, the Delvotest® SP-NT did not detect them. This result is in agreement with the data reported from the studies of Zhou et al. (2007) and Johny et al. (2010). They determined that combinations of natural antimicrobial compounds were more effective at reducing populations of *Salmonella typhimurium* than treating with one compound alone.

Holstein and Jersey cows were selected for this study because Jersey cows typically have greater concentrations of MF and MP, which could affect the outcome of the Delvotest ® SP-NT.   In a study done by Andrew (2000), the specificity rates (rate of truly negative samples that were found negative) were lower when milk collected from Jersey cows was evaluated in comparison to milk evaluated from Holstein cows. Greater concentrations of MF and MP may interfere with the performance of the screening tests either by inhibiting the growth of the test organism for microbial inhibition tests or affecting the chemical reactions in the specific residue screening tests. Sischo and Burns (1993) reported that an increased SCC was associated with an increase in the rate of false positive outcomes for the Delvotest-P® (Sischo and Burns, 1993). This association was also found in a study by Van Eenennaam et al. (1993). The researchers evaluated the efficacy of four residue screening tests, including the IDEXX CITE® Probe (β-lactam) and the Delovtest-P®, for cows recovering from mastitis. Milk containing SCC of greater than 10^6 cells/mL was associated with a greater number of false-positive outcomes for both the CITE® SNAP and the Delovtest-P® screening tests (Van Eenennaam, 1993). In

the current study, elevated SCC did not interfere with the ability of the Delvotest® SP-NT to detect the natural antimicrobials; and therefore, the screening tests may be useful in identifying these compounds in milk across a wide range in MF, MP, and SCC.

CONCLUSION

The results of this study support the hypothesis that microbial inhibition tests can detect the natural antimicrobials at their MIC, for all the compounds evaluated, and concentrations below the MIC, for all the compounds with the exception of *trans*-cinnamaldehyde. The treatment of mastitis constitutes the majority of antibiotic usage on conventional dairy farms. The treatment of IMM infusion of antibiotics is prohibited on organic facilities; therefore, "natural antimicrobials" such as the compounds in this study may have the potential to be efficacious, meet organic standards, and be evaluated for approval by the FDA. This study has clearly demonstrated that the microbial inhibition antibiotic residue screening test, the Delvotest® SP-NT, can detect these natural antimicrobials and indicate that these compounds may, like nisin affect microbial activity of cultured milk products. Microbial inhibition tests, like the Delvotest® SP-NT, can provide producers the means of ensuring the quality of their products. These findings may be useful to producers, regulatory agencies, and processing plants for assurance of safety and quality of milk produced by organic dairy facilities. Further *in vivo* research is needed to determine the effect of these natural antimicrobials on milk quality and cultured dairy products.

Table 1. List of antimicrobials, their commercial reference numbers, and their minimum inhibitory concentration (MIC).

Antimicrobial	Reference no.	MIC (mM)
Caprylic Acid	153753[1,2]	50.0
Trans-cinnamaldehyde	239968[1,3]	7.57
Eugenol	E51791[1,3]	36.5
Carvacrol	282197[1,3]	33.3
Thymol	T0501[1,3]	39.9
Nisin[4]	___	2.00[5,6]
Phyto-mast[®][7]	___	___

[1] Sigma-Aldrich Chemical Company, St. Louis, MO
[2] MIC determined by Nair et al., 2005
[3] MIC determined by Ananda Baskaran et al., 2009
[4] ImmuCell Corporation, Portland, Maine
[5] MIC determined by Piper et al., 2009
[6] MIC expressed in nM
[7] Penn Dutch Cow Care, Narvon, PA

Table 2. Somatic cell count (SCC), milk fat and milk protein contents for Holstein and Jersey cows that were sampled for the detection of caprylic acid, *trans*-cinnamaldehyde, eugenol, carvacrol, thymol, and phyto-mast® in milk by the Delvotest® SP-NT.

Variable	Mean	Minimum	Maximum
Holstein (n=59)			
SCC (x 10^3/mL)	53	4	400
Milk fat (%)	3.20	2.10	4.70
Milk protein (%)	2.80	2.40	3.40
Jersey (n=37)			
SCC (x 10^3/mL)	107	3	841
Milk fat (%)	4.30	3.00	5.40
Milk protein (%)	3.40	2.60	4.10

Table 3. Sensitivity rates for the Delvotest® SP-NT using milk spiked with caprylic acid, *trans*-cinnamaldehyde (TC), carvacrol, eugenol, and thymol at three different concentrations including the minimum inhibitory concentration (MIC), the detection limit (DL), and greatest concentration below the detection limit (BDL).

Antimicrobial	Inhibitory activity measures	Concentration (mM)	Number positive	Sensitivity rate	Lower CI[1]	Upper CI[2]
Caprylic Acid	MIC	50.0	28	0.93	0.76	0.99
	DL	20.0	28	0.93	0.76	0.99
	BDL	10.0	1	0.03	0.001	0.19
TC	MIC	7.57	30	1.0	0.86	1.0
	BDL	2.27	0	0	0	0
Carvacrol	MIC	33.3	30	1.0	0.86	1.0
	DL	9.98	29	0.96	0.81	0.99
	BDL	3.33	1	0.03	0.001	0.19
Eugenol	MIC	36.5	30	1.0	0.86	1.0
	DL	12.2	30	1.0	0.86	1.0
	BDL	3.05	1	0.03	0.001	0.19
Thymol	MIC	39.9	30	1.0	0.86	1.0
	DL	16.6	30	1.0	0.86	1.0
	BDL	6.65	0	0	0	0

[1] Lower 95% confidence interval
[2] Upper 95% confidence interval

Table 4. Sensitivity rates for the Delvotest® SP-NT using milk from Jersey (n=15) and Holstein cows (n=15) spiked with nisin and phyto-mast® at the minimum inhibitory concentration (MIC), the detection limit (DL), and below the detection limit (BDL).

Antimicrobial	Inhibitory activity measures	Concentration (nM)	Number positive	Sensitivity rate	Lower CI[1]	Upper CI[2]
Nisin	MIC	2.00	30	1.0	0.86	1.0
	DL	0.005	30	1.0	0.86	1.0
	BDL	0.0025	0	0	0	0
Phyto-mast®	DL	125[3]	30	1.0	0.86	1.0
	BDL	62.5[3]	1	0.03	0.001	0.19

[1] Lower 95% confidence interval
[2] Upper 95% confidence interval
[3] Expressed in µL/mL of milk

Table 5. Minimum inhibitory concentration (MIC), detection limit (DL) and percent DL relative to MIC (% DL:MIC) for the Delvotest® SP-NT using milk spiked with caprylic acid, *trans*-cinnamaldehyde, eugenol, carvacrol, thymol, and nisin (expressed in mM).

Antimicrobial	MIC	DL	% DL:MIC [1]
Caprylic Acid	50.0	20.0	60.0
Trans-cinnamaldehyde	7.57	7.57	0
Eugenol	36.5	12.2	66.9
Carvacrol	33.3	9.98	70.0
Thymol	39.9	16.6	58.4
Nisin	2.00[2]	0.005[2]	99.8

[1] DL relative to MIC for each compound. Calculated as (MIC –DL) / (MIC) x 100
[2] Expressed in nM

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