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ANTIBACTERIAL ACTIVITY OF DIFFERENT ORGANIC HONEYS AGAINST FOOD PATHOGENIC BACTERIUM *CLOSTRIDIUM PERFRINGENS*

DJAMILA OINAALA, ULRIKE LYHS AND CARINA TIKKANEN-KAUKANEN

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PREFACE

This study was written based on the thesis by Djamila Oinaala, written for the fulfillment of the requirements for the Bachelor degree in Nutrition and Food Quality. The actual research was executed as exchange training in Ruralia Institute, University of Helsinki, Mikkeli. The research work was carried out during the spring 2012 in collaboration with the Mikkeli University of Applied Sciences. *C. perfringens* type A is known to cause a broad spectrum of human and animal diseases. It is one of the third most common causes of foodborne illnesses. Antibiotic resistant *C. perfringens* strains are becoming major health concern. Antibiotic resistance is a serious problem worldwide, and it has made the search for new antimicrobial compounds more important. Honey has been used as a traditional medicine for centuries. Many in vitro studies have revealed antimicrobial activity of different honeys against several bacterial strains, including antibiotic resistant bacteria.

This study had two different parts related on virulence of *C. perfringens*. In the first part of the study antimicrobial activity of organic honeys were investigated against *C. perfringens*. Organic honeys were chosen for the study in order to use of organic honeys as preservatives in organic food products. Organic honeys have not been investigated before as regards their antimicrobial activity. Although food products in Finland are commonly considered to be pure the main difference compared to non-organic honeys is the guaranteed purity as regards the contaminating agents, drugs, pesticides and other contaminants from the environment. Production of organic honey is regulated by EU-directive and in Finland organic honey production is controlled by Evira (Finnish Food Authority). In the present study antimicrobial activity of organic honeys against *C. perfringens*, a food poisoning bacterium, was found for the first time. This is also the first report on antibacterial activity of organic honeys. The present study gives rationale for further studies on antimicrobial activity of organic honeys against food poisoning bacteria including characterization of the antimicrobial components. Considering organic honeys as preservatives in food products, especially in organic food, it would be important to characterize the antimicrobial components and evaluate the effect of heat treatments on antimicrobial activity. Clinical intervention would be valuable to confirm the possible protective effect of the studied honeys against *C. perfringens* infections in humans and further against other food poisoning bacteria. However, one has to keep in mind that honey is not allowed to give for children under 1-year-old, because it may contain botulinium spores. In the second part of the work *C. perfringens* isolates from healthy broilers and from broilers suffering from necrotic enteritis (NE) were analyzed by polymerase chain reaction (PCR) method in order to determine the presence of the NE toxin B (NetB), a novel toxin that has been strongly associated with the pathogenesis of NE. These results imply that although there is a clear association between the presence of netB toxin gene and development of NE there may be other virulence factors that are produced by these netB-negative disease-producing isolates. The role of NetB in the introduction of NE needs further investigations.

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CONTENTS

ABSTRACT

Clostridium perfringens is one of the most common causes of food poisonings and is known to cause human and animal diseases. It has been reported that *C. perfringens* has developed resistance to antibiotics. Antimicrobial properties of honey have been studied in order to confirm its action as a potential antimicrobial agent and also as a potential food preservative through inactivation, growth delay or growth prevention of food pathogenic microorganisms.

This study had two different parts related on virulence of *C. perfringens*. In the first part of the study antimicrobial activity of five multifloral organic honeys from different parts of Finland and one multifloral honey originated from Argentina and Hungary were investigated against *C. perfringens*. Honeys were tested at the concentrations of 50% (w/v), 25% (w/v), 20% (w/v), 15% (w/v) and 10% (w/v). For the antimicrobial assessment a disc diffusion method was used and zone of inhibition was expressed as diameter. The minimum concentrations (minimum inhibitory concentration, MIC) needed for inhibition of bacterial growth were determined for honeys showing activity in dilutions of 50%. Four of the studied honeys showed good inhibitory activity (diameter >8 mm) compared to control sugar solution (diameter of 6.1 ± 1.5) against *C. perfringens* at the concentration of 50 % (w/v). All the active honeys were of Finnish origin. The broadest zone of inhibition was induced by North Carelien multifloral organic honey with willow herb as the main floral source (referred here as F) (diameter of 14,3 mm ± 0.6), followed by other North Carelien multifloral organic honey (diameter of 11 mm ± 2) with clover as the main floral source (referred here as E). In terms of MIC values North Carelien organic honey F was also the most efficient honey against C. perfringens and showed moderate antibacterial activity (diameter of 7-8 mm) even at 10% (w/v). The other active honeys showed moderate inhibitory activity in the dilution of 15%. To our knowledge this is the first report on the antimicrobial activity of organic honeys and antimicrobial activity of honey against *C. perfringens*. (Oinaala et al 2015)

In the second part of the work *C. perfringens* isolates from healthy broilers and from broilers suffering from necrotic enteritis (NE) were analyzed by polymerase chain reaction (PCR) method in order to determine the presence of gene coding the NE toxin B (NetB), a novel toxin that has been strongly associated with the pathogenesis of NE. The *netB* toxin gene was detected in 30% isolates from broilers with necrotic enteritis and in 32.5% isolates from healthy broilers. In this study the presence of *netB* gene gives not enough information for predicting the virulence of *C. perfringens* isolates, thus, further investigations are needed.

Key words: Organic honey, antimicrobial activity, foodborne pathogenic bacteria, netB toxin, *netB* gene, *C. perfringens,* food poisoning

TIIVISTELMÄ

Clostridium perfringens on yksi tärkeimmistä ruokamyrkytyksen aiheuttajista ja se aiheuttaa tautia sekä ihmisille että eläimille. *C. perfringens* on kehittänyt vastustuskykyä antibiooteille. Hunajan antimikrobisia ominaisuuksia on tutkittu tarkoituksena selvittää sen kykyä toimia antimikrobisena aineena ihmisille ja eläimille. Hunajalla on potentiaalia myös ruoan säilönnässä, se voi viivästyttää tai estää ruokaa pilaavien bakteerien kasvua.

Tässä tutkimuksessa oli kaksi eri osaa liittyen *C. perfringens*-bakteerin taudinaiheuttamiskykyyn, virulenssiin. Ensimmäisessä osassa tutkittiin luomu-monikukkahunajien antimikrobista aktiivisuutta *C. perfringens*-bakteeria vastaan. Tutkimuksessa oli mukana viisi luomuhunajaa, jotka oli tuotettu eri puolella Suomea sekä yksi Argentiinassa ja Unkarissa tuotettu luomuhunaja. Hunajat tutkittiin 50% (w/v), 25% (w/v), 20% (w/v), 15% (w/v) ja 10% (w/v) pitoisuuksissa. Antimikrobisuus tutkittiin kiekkodiffuusiomenetelmällä ja muodostunut estorengas ilmoitettiin halkaisijan leveydellä. Pienin bakteerin kasvua estävä pitoisuus (minimal inhibitory concentration, MIC) määritettiin hunajille, jotka olivat aktiivisia 50 % pitoisuudessa. Tutkituista hunajista neljä osoitti hyvää estävää vaikutusta (halkaisija > 8mm) verrattuna sokeriliuokseen, joka toimi negatiivisena kontrollina (estorengas 6.1 mm ± 1.5). Kaikki antimikrobista aktiivisuutta omaavat hunajat olivat suomalaisia luomuhunajia. Laajimman estorenkaan aiheutti Pohjois-Karjalan luomu-monikukkahunaja (F), joka oli pääosin horsmahunajaa (halkaisija 14,3 mm ± 0.6). Pohjois-Karjalan luomu-monikukkahunaja (E), jossa pääasiallinen kukkalähde oli apila, aiheutti seuraavaksi suurimman estorenkaan (11 mm ± 2). MIC-arvo Pohjois-Karjalan hunajalle F oli tehokkain, se osoitti aktiivisuutta vielä 10% pitoisuudessa (estorengas 7-8 mm). Muiden aktiivisten hunajien MIC-arvot olivat 15%. Tietämyksemme mukaan tämä on ensimmäinen raportti liittyen luomuhunajien antimikrobiseen aktiivisuuteen ja hunajan antimikrobiseen aktiivisuuteen *C. perfringens*-bakteeria vastaan.

Tutkimuksen toisessa osassa analysoitiin *C. perfringens*-bakteerikantoja polymeraasiketjureaktio (PCR)-menetelmällä. *C. perfringens*-bakteerikannat eristettiin sekä terveistä että kuolioiseen suolistotulehdukseen (nekroottinen enteriitti, NE) sairastuneista kananpojista. Eristetyistä bakteerikannoista määritettiin NE-toksiini B (NetB)-geeni, jonka esiintyminen on vahvasti liitetty NE-tulehdukseen. NetB-toksiinia koodaava geeni löydettiin sekä NE -tulehdukseen sairastuneista kananpojista eristetyistä kannoista (30% kannoista) että terveistä kananpojista eristetyistä kannoista (32.5%). Tässä tutkimuksessa *netB*-geenin läsnäolo ei antanut riittävästi tietoa *C. perfringens*-bakteerikantojen taudinaiheuttamiskyvystä, joten uusia tutkimuksia tarvitaan.

ABBREVIATIONS

BC	Before Christ
BHI	Brain heart infusion
EVIRA	Finnish Food Safety Authority
MGO	Methylglyoxal
MIC	Minimum inhibitory concentration
NE	Necrotic enteritis
PCR	Polymerase chain reaction

SYMBOLS

a_w	Water activity
µg	Microgram
µl	Microliter
w/v	Weight per volume
v/v	Volume per volume
Eh	Oxidation reduction potential
CPE	Enterotoxin gene
Na^+	Sodium ion
Cl^-	Chloride ion
Ca^{2+}	Calcium ion
°C	Celsius degrees
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate

1 INTRODUCTION

Food market trends are constantly changing. Nowadays consumers have become more demanding and more aware in relations of food that they eat. They search for food of higher quality which means less extreme treatments and/or additives and more foods with freshlike attributes (Gould, 1996). Consumer demands can be satisfied, with some adjustments or reductions in conventionally used preservation techniques (Gould, 1992). These can be possible if the safety and quality of foods are based on substantial improvements in traditional preservation methods or on the use of emerging technologies for food processing (Gould, 2002). These changes have important and significant implications from a microbiological point of view. Microbial growth in foods has negative consequences such as consumer hazards due to the presence of pathogenic organisms or microbial toxins, and it may also result in economic losses as a result of spoilage (Davidson, 2001). Inactivation, growth delay, or growth prevention of spoilage and pathogenic microorganisms are the first steps for food preservation. Antibiotics have been associated with development of antibiotic resistant bacteria that can cause foodborne illness. The use of antibiotics in food production is restricted and must be minimized.

C. perfringens type A is known to cause a broad spectrum of human and animal diseases. It is one of the most common causes of foodborne illness in Europe, Japan and the United States (Wen *et al.,* 2004). Antibiotic resistant *C. perfringens* strains are becoming a major health concern. A study by Teuber (1999) indicated that copious use of antibiotics in agriculture is promoting a large antibiotic resistance problem in foodborne pathogens, including *C. perfringens*. According to the Public Health Agency of Canada strains of *C. perfringens* have developed resistances of penicillin, tetracycline, erythromycin, chloramphenicol, metrondiazole, and clindamycin. Because of the rise of drug resistant strains of *C. perfringens* new antimicrobials are needed.

It is now widely accepted that honey has antimicrobial activity and that this is dependent upon a variety of different modes of action (Molan, 1992). Many *in vitro* studies have revealed the ability of honey to inhibit growth of wide range of skin colonizing and food-borne bacterial species, including antibiotic resistant bacteria. However, there is only a small report (Chen *et al.,* 2000; Taormina *et al.,* 2001; Melissa *et al.,* 2004) about the ability of honey to inhibit growth of food spoilage organisms or foodborne pathogenic bacteria. If honey can slow or stop the growth of spoilage organisms or food pathogens, which are the main objectives of food preservation, then its incorporation into foods as a preservative can be explored.

The *C. perfringens* necrotic enteritis toxin B (*NetB*) was recently proposed as a new key virulence factor for the development of NE in broilers (Keyburn et al.). Although there is a clear association between the presence of *netB* toxin gene and development of NE there may be other virulence factors that are produced by these *netB*-negative disease-producing isolates. The NetB toxin may not be an obligate requirement for poultry *C. perfringens* virulence and at least the presence of *netB* may not be essential for the disease process in all *C. perfringens* isolates. Today the role of *NetB* in the introduction of NE is unclear and needs further investigations.

2 REVIEW OF THE LITERATURE

2.1 ANTIMICROBIAL ACTIVITY OF HONEY

Honey has been used as a medicine in ancient times in many cultures and was used against various diseases, or as a preservation method. Nowadays a large amount of research work has been done on the antimicrobial activity of honey (Molan, 1992). The intensive study on the antimicrobial activity of honey commenced with Dold *et al.* (1937) in Germany. It was discovered that honey, especially when diluted with water, was effective in killing certain bacteria, and they attributed the effect to "inhibine". Since then there have been many reports. The reports are mostly related with the antimicrobial activity spectrum of the honey, which species of micro-organism were sensitive to the action of honey or comparing the potency of the different types of honey and its action against one or more species of bacteria. Also there have been many investigations of the nature of the antibacterial substances present (White *et al.,* 1963; Molan, 1992). It has been shown *in vitro* that unheated honey has broad-spectrum antibacterial activity. It has been well documented that honey has a bacteriostatic and bactericidal effect against various species of both gram positive and gram negative bacteria and it has antifungal effect. Because of beneficial actions of honey against wound infections it is used *in vivo* and licensed honey products are widely used in wound care.

2.1.1 HISTORY OF USE OF HONEY

For 4000 years medicinal, pharmaceutical and health-giving properties of honey have been described. Traditional medicine used honey for treating many disorders, being soothing, nutritious, laxative and regulatory. During the Biblical era honey received religious endorsement by Christianity (Judaism). God promised to give "a land flowing with milk and honey" to people of Israel (Zumla and Lulat, 1989).

Hippocrates (3rd and 4th centuries BC) prescribed a simple diet, favoring honey given asoxymel (vinegar and honey) for pain, hydromel (water and honey) for 'thirst', and a mixture of honey, water and various medicinal substances for acute fevers (Zumla and Lulat, 1989). He considered that honey was a very good expectorant because "it causes spiting".

Babylonian and Assyrian medicine used honey in prescriptions for treating disorders of eyes and ears (Crane, 1999). Susruta (c.1400 BC), the famous Ayurvedic surgeon, have used paste of honey and butter enriched with barley and four herbs to treat wounds (Crane, 1999).

In Ancient Egypt honey was included in 102 of several hundred internal remedies listed in the Ebers papyrus. It was used especially for treating respiratory disorders with symptoms such as catarrh, cough and asthma (a wheezing cough), and constipation, diarrhea and intestinal worms, also for disorders of the digestive tract including the cardia (the oesophageal orifice to the stomach).

In ancient times honey was used also for preserving a human corpse. Bodies were "smeared with wax and buried in honey" during the reign of Sargon during 2371-2316 BC. The Babylonians used honey for preserving corps before 1100 BC. In the 400 BC Democritus in Greece was reported to have been buried in honey (Crane, 1999).

Ilb el-Beithar, a physician born in Malaga at the end of the 1100s, wrote about honey in his book of simples (herbal remedies): "Honey dispels humors (body fluids), relaxes the bowels, is helpful in treating dropsy, preserves flesh and prevents putrefaction, stimulates de appetite, is good against facial tic. Mixed with sesame oil and boiled wine it is used as an emetic when poison has been swallowed. It is the best treatment for gums, and for teeth, which it also whitens, it gives good results with tonsillitis and stimulates coitus and taken with water it cleanses intestinal ulcers and enhances the effect of medication. Honey that has not been heated is useful against stomach chills, swelling of the intestines, and also stomach disorders due to the pituitary gland". (Crane, 1999)

2.1.2 PROPERTIES OF HONEY THAT RESTRICT MICROBIAL GROWTH

A study conducted by Molan (1992) pointed out that number of characteristics of honey contribute to its antimicrobial activity. Honey is a supersaturated sugar solution, 70-80% % being a mixture of fructose and glucose with a low water activity (a_w) (15-21% by weight), which means that there is little water available to support the growth of bacteria and yeast. Many species of bacteria need 0.94-0.99 a_w while ripened honey has 0.56-0.62 a_w. Its natural acidity, pH between 3,2 and 4,5, inhibits many pathogens. The pH and antibacterial hydrogen peroxide can be raised, if honey is diluted especially with body fluids (Molan, 2001).

The hypopharyngeal glands of worker bees secrete the enzyme glucose oxidase into nectar, when the bees are processing it into honey. Glucose oxidase is activated by diluting of honey, when hydrogen peroxide is produced. Hydrogen peroxide it is one of the most important agent responsible for the antimicrobial activity of honey (Molan, 2001).

Honeys may contain various antibacterial substances, like flavonoids and other phenolic compounds that presumably produced by certain species of plants possessing antioxidant activity. Estevinho *et al.* (2008) have shown that phenolic compounds extract obtained from dark honeys have stronger antioxidant activity; they also reported that the difference in the phenolic compounds depends on of the geographic origin (flora predominance) of the honey.

Fujiwara *et al.* (1990) have isolated honey bee secretion, bee-defensin-1, from royal jelly, which is food prepared by bees for queen larvae. (Kwakman *et al.*, 2008) demonstrated that Revamil medical grade honey contains also the bee defensin-1 protein, and that this protein substantially contributes to the bactericidal activity of honey.

Methylglyoxal (MGO) is the aldehyde form of pyruvic acid. Mavric *et al.* (2008) have reported that MGO is directly responsible for the antibacterial activity of Manuka honey. Recent studies have, however, shown that although methylglyoxal was a major bactericidal factor in manuka honey, there are still other several unknown factors that contribute on antimicrobial activity of manuka honey (Kwakman *et al.*, 2011).

2.1.3 HONEY AGAINST FOODBORNE PATHOGENIC AND SPOILAGE BACTERIA

There is a great interest in controlling the growth or eliminating foodborne pathogens using natural antimicrobials. The use of the drugs, whether therapeutically or sub therapeutically, results in increase of the proportion of antibiotic-resistant bacteria. Many foodborne bacteria carry genes that confer antibiotic resistance on mobile genetic elements, which have the ability to leave and enter bacterial cells (Duffy *et al.,* 2005).

Due to its antimicrobial properties, honey may serve as a natural food preservative. Previous research has demonstrated preservative power of honey by reducing enzymatic browning of fruits (Chen *et al.,* 2000) and preventing lipid oxidation in meat (McKibben *et al.,* 2002) which work has confirmed the antioxidant capacity of some honeys.

Taormina *et al.* (2001) found that both peroxide and non-peroxide components in honey affected the growth of six food pathogens, *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Shigella sonnei*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus*. Melissa *et al.* (2004) have demonstrated that honey was shown to be capable of inhibiting the growth of both spoilage microorganisms *(Alcaligenes faecalis, Aspergillus niger, Bacillus stearothermophilus, Geotrichum candidum, Lactobacillus acidophilus, Penicillium expansum, Pseudomonas fluorescens)* and foodborne pathogens (*Bacillus cereus, Escherichia coli O157:H7, Listeria monocytogenes, Salmonella enterica ser. Typhimurium*) and could be considered for use as a food preservative under appropriate conditions.

2.1.4 THE ROLE OF HONEY IN THE MANAGEMENT OF WOUNDS

The early use of honey in wound treatment has recently received attention from several medical scientists. The ancient Egyptians, Assyrians, Chinese, Greeks and Romans employed honey for wounds and gut related diseases (Zumla and Lulat,1989).

Honey is being used with success on infections not responding to standard antibiotic and antiseptic therapy. It is effective in rapidly clearing up infection and promoting healing (Molan, 2001). Honey's antibacterial activity in wound dressing is partly due to the high osmolarity created by its sugar content. When honey is applied topically to wounds, osmosis would be expected to draw water from the wound into the honey, helping to dry the infected tissue and reduce bacterial growth. Honey's antimicrobial activity is also due to hydrogen peroxide. Low concentrations of this known antiseptic are effective against infectious bacteria and can play a role in the wound healing mechanism (Molan, 2001) and in stimulation and proliferation

of peripheral blood lymphocytic and phagocytic activity (Tonks *et al.,* 2001). In some honeys, there are additional antibacterial factors such as bee defensin 1 protein in Revamil honey (Kwakman *et al.,* 2010). Methylglyoxal, the antibacterial factor detected first in Manuka honey gives honey more potent antibacterial action making the honey effective especially in wound healing (Mavric *et al.*, 2008)., althoug recent research has shown that in Manuka honey there additional antimicrobial factors that are unkown (Kwakman et al., 2011).

A number of laboratory studies have demonstrated the significant antibacterial activity of honey against wound infecting bacteria. Molan (1992) used different concentrations of honey ranging from 1.8% to 11% (v/v), and they achieved complete inhibition of the major wound-infecting species of bacteria. Studies conducted by Cooper *et al.* (1999) reported complete inhibition of 58 strains of coagulase-positive *Staphylococcus aureus* isolated from infected wounds with 2%-4% (v/v) of honey and complete inhibition of 20 strains of *Pseudomonas* isolated from infected wounds with 5.5%-8.7% (v/v) of honey.

Branikl (1981) reported that infected wounds dressed with honey became sterile in 3-6 days. Today Revamil and Manuka medical grade honeys are medical honeys mostly used in wound management because of their additional antimicrobial mechanisms.

2.2 *CLOSTRIDIUM PERFRINGENS*

2.2.1 MORPHOLOGY AND PHYSIOLOGY CHARACTERISTICS

C. perfringens is a Gram-positive, rod-shaped and non-motile bacterium. It is mesophilic with a growth optimum between 37°C and 45°C (James *et al.,* 2005). It has the ability to form spores which are resistant to environmental stresses (Labbe, 1989). *C. perfringens* is a strictly anaerobic bacterium but is able to survive when exposed to oxygen for short periods of time (Labbe, 1989).

2.2.2 SURVIVAL AND GROWTH CONDITIONS OF *C. PERFRINGENS*

C. perfringens spores are very heat resistant. Incomplete cooking of foods may not only fail to kill *C. perfringens* spores but can actually favour the development of *C. perfringens* type A food poisoning. The resistant properties of *C. perfringens* spores are influenced by environmental factors (e.g. the heating medium) and by genetic factors. Vegetative cells of *C. perfringens* are also somewhat heat tolerant. They are not notably tolerant of cold temperature, including either refrigeration or freezing conditions in contrast to *C. perfringens* spores that are cold resistant. Growth rates of *C. perfringens* rapidly decrease at temperature below 15°C, with no growth occurring at 6°C. (Labbe, 1989)What is the difference between Labbe, 1989 and Labbe, 1989a? In the list is only Labbe, 1989. lease, check!

C. perfringens requires only relatively modest reductions in oxidation-reduction potential for growth. If the redox potential (Eh) of the environment is suitably low for initiating growth, *C. perfringens* can modify the Eh by producing reducing molecules, such as ferredoxin to produce more optimal growth conditions. (Labbe, 1989)

The growth of *C. perfringens* is also pH sensitive. The optimal growth of this bacterium occurs at pH 6 to 7, a pH range similar to that of most meat and poultry products. The growth is severely limited at pH ≤ 5.0 and 8.3≥ (Smith, 1972). In cases of foodborne illness some inactivation of cells occurs during stomach passage due to the low pH levels encountered. In foodborne outbreaks, cells are ingested along with large volumes of food, which provides some protective, buffering effect against stomach acidity. It has been reported that acid exposure (pH 2.0, 30 minutes) enhances enterotoxin formation although others have been unable to produce this effect (Labbe, 2006).

The effectiveness of curing salts (for example, sodium nitrate) for limiting *C. perfringens* growth in foods is unsolved (Labbe, 1989). Some studies have indicated that the concentrations of curing salts needed to significantly affect on *C. perfringens* growth exceed acceptable levels for food products. The growth inhibition of *C. perfringens* requires at least 6 to 8% of NaCl and 10.000 ppm of $NaNO_3$ or 400 ppm of $NaNO_2$.

2.2.3 CHARACTERISTICS OF THE TOXINS

C. perfringens is divided into five toxin types (A-E) based on the production of four major toxins alpha toxin (α), beta toxin (β), iota toxin (ι) and epsilon toxin (ε), (Table 1) where each type carries a different combination of the toxin genes. However, this bacterium also produces at least 13 other toxins such as enterotoxin (CPE), theta toxin, beta2 toxin and *netB* toxin (Petit *et al.,* 1999, Keyburn *et al.,* 2008).

Toxin typing of *C. perfringens* has traditionally involved laborious toxin antiserum neutralization tests in mice, but the development of PCR-based schemes permitting toxin genotyping of *C. perfringens* isolates has greatly simplified toxin typing (Daube *et al.,* 1996).

Table 1. Distribution of the four major lethal toxins among types of *C. perfringens* (according to Labbe and Juneja, 2006)

Type	Lethal toxin			
	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Alpha toxin, a protein, is produced by all types of *C. perfringens* and is hemolytic, necrotizing and lethal. It was previously thought to be associated as the major virulence factor in NE in chickens (Al-Sheikhly *et al.,* 1977) A recent report using an alpha-toxin mutant demonstrated that alpha toxin was not necessary for the pathogenesis of the disease (Keyburn, *et al.,* 2006). Although alpha toxin has been implicated in many diseases, the exact mode of action of the toxin is still unclear.

Found in *C. perfringens* type B and C strains, **beta-toxin** is consisted of a single polypeptide chain of 336 amino acids with a predicted molecular weight of approximately 34.9 kDa. Beta-toxin is a major virulence factor in causing enterotoxaemia and NE of many animal species including sheep, lambs, and fowl and it is also the causative agent of human enteritis necroticans or pig-bel (Huang, 2007). Pig-bel is caused by the consumption of under-cooked meat products contaminated with *C. perfringens* type C spores (Huang, 2007).

Epsilon-toxin is considered the most potent clostridal toxin after botulinum and tetanus neurotoxins (Huang, 2007). Produced by type B and D strains only, it causes animal dysentery and enterotoxemia, respectively (Sterne *et al.,* 1964; Huang, 2007). The mode of action of epsilon-toxin is still unclear. However, it is known that epsilon-toxin can increase vascular permeability in the brains, kidneys and intestines (Huang, 2007).

Iota toxin is a binary toxin found in *C. perfringens* type E (Carman *et al.*, 1997). *C. perfringens* type E has been identified only occasionally as the cause of diarrhea in animals particularly in domesticated livestock (Carman *et al.*, 1997).

Some type A strains produce **enterotoxin** which causes diarrhea in humans and in various domestic animals (Baums *et al.,* 2004). It is synthesized by sporulating cells in association with late stages of sporulation. Conditions that favor sporulation also favor enterotoxin production. Enterotoxin is heat-labile (inactivated at 74 °C) and can be detected in contaminated food, if not heated properly, and in feces.

NetB toxin is cytotoxic for avian cells and might be a key virulence factor in *C. perfringens* strains that cause avian NE (Keyburn *et al.*, 2010). *NetB* has 38% protein sequence identity to the beta-toxin of *C. perfringens*, which causes mucosal necrosis of the small intestine in humans and animal (Keyburn *et al.,* 2008). The *netB* gene is located on a plasmid and encodes a poreforming toxin, which perforates the plasma membrane and thereby damage host cells (Keyburn *et al.,* 2008, 2010). Some authors consider NetB the most important bacterial virulence factor for development of NE although both *netB* positive and negative strains have been found associated with NE (Timbermont *et al.,* 2011).

2.2.4 RESERVOIRS OF *C. PERFRINGENS*

C. perfringens is ubiquitous in the environment, being found in soil, in decaying organic matter and as a member of the normal intestinal flora of many humans and animals. It causes a broad spectrum of human and animal diseases. *C. perfringens* level in normal human feces is usually 10^3–10^4 CFU/g. Up to 39 different *Clostridium* species have been isolated from human feces. In foodborne illness, the outbreak strains are ingested and present in feces at higher levels than are resident strains. *C. perfringens* type A is part of the microflora of the soil. It also can be present in dust. The association of *C. perfringens* with the intestinal tract has led to its use as an indicator of fecal pollution of water. It has similarly been used as an indicator of movement of sewage sludge on the ocean floor, where extreme conditions preclude the use of traditional indicators of water pollution such as coliforms and fecal streptococci. There are many surveys of the incidence of *C. perfringens* in raw and processed foods. Meats are contaminated by slaughtering animals or by subsequent contamination of slaughtered meat from containers, handlers or dust. Furthermore, *C. perfringens* also exists in herbs and spices.

2.2.5 FOOD POISONING

C. perfringens has been recognized as a cause of foodborne illness since the late 1940s. *C. perfringens* type A food poisoning is ranked as the third most commonly reported agent of human foodborne diseases. The most common food vehicles for *C. perfringens* type A foodborne illness are meats (notably beef and poultry and also meat-containing products). Clinical symptoms are characterized by acute abdominal pain and diarrhea. However, nausea, fever and vomiting are rare. The symptoms are generally developed about 8 to 16 h after ingestion of contaminated food (food containing > 10^6 to 10^7 vegetative cells per gram of food). If the food are improperly prepared or cooked, spores of *C. perfringens* are able to survive and germinate in the nutrient rich environment, leading to the proliferation of large number of *C. perfringens* cells. Once the contaminated food is ingested, the majority of the bacteria might be killed by the acidic condition of the stomach; however, a small number of the population will survive and enter into the intestine.

3 AIMS OF THE PRESENT STUDY

This study had two different parts related on virulenceof *C. perfringens*. In the first part of the study antimicrobial activity of six different organic honeys was investigated against *C. perfringens*. For the antimicrobial assessment and the determination of minimum inhibitory concentration a disc diffusion method were used. Honeys were tested at the concentrations of 50% (w/v), 25% (w/v), 20% (w/v), 15% (w/v) and 10% (w/v). In the second part of the work *C. perfringens* isolates from healthy broilers and from broilers suffering from NE were analyzed by polymerase chain reaction (PCR) method in order to determine the presence of the *netB* gene.

4 ANTIBACTERIAL ACTIVITY OF HONEY

4.1 MATERIALS AND METHODS

4.1.1 ORGANIC HONEY SAMPLES

One of the studied organic multifloral honeys was produced in Hungary and Argentina (referred here as A). All the other honeys examined (referred as B-F) were produced in different parts of Finland. Multifloral organic honey produced in Eastern Finland, Haarajärvi, North Karelia (referred here as B), mutltifloral organic honey was obtained from Central Finland, from Korpilahti, Jyväskylä, (referred here as C), multifloral organic honey from Eastern Finland Ihastjärvi, South Savonia (referred here as D) were studied. Organic multifloral honeys A, B, C and D were purchased from the local supermarkets in March of 2012. Two of the studied honeys were provided by local beekeepers, North Karelia organic honey from Eastern Finland Hoilola, Joensuu (referred here as F) and organic honey from Eastern Finland Ilomantsi, North Karelia (referred here as E). Honey samples provided by local beekeepers were obtained in ready commercial package. All the studied honeys were multifloral and contained different floral sources. The main floral source was reported to the Finnish honeys B, C, D, E and F. The main floral sources in honeys B and C were wild raspberry (genus Rubus), willow herb (*Epilobium angustifolium)*, lingonberry *(Vaccinium vitis-idea)* and bilberry (*Vaccinium myrtillus*). In honey D the main floral sources were wild raspberry (genus Rubus) and lingonberry (*Vaccinium vitis-idea*). In honey E the main floral source was clover (genus Trifolium) and for honey F willow herb (*Epilobium angustifolium*). Honey D was treated by quick heat treatment at 50 °C. All the other studied honeys were untreated.

4.1.2 BACTERIAL STRAIN AND CULTURE CONDITIONS

The bacterium used in this research was a *C. perfringens* type A strain (α-toxin positive and *netB* negative) isolated from turkey with NE infection (Figure 1.) (CLO 555, strain collection of the Finnish Food Safety Authority Evira). The strain was cultured on blood agar containing 5% defibrinated sheep blood (this is ok like it is) and incubated anaerobically (AnaerobicCult A, Merck, Darmstadt, Germany) at 37°C for 24 to 48 h.

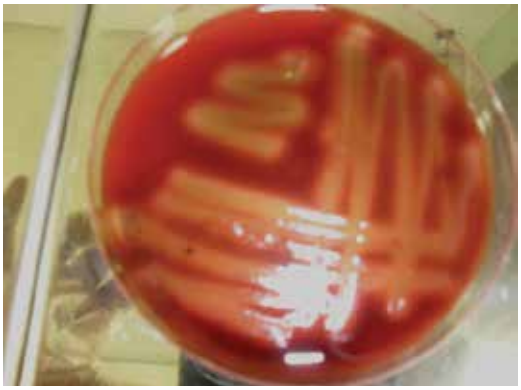

Figure 1. *C.perfringens* type A strain (CLO 555)

4.1.3 PREPARATION OF THE HONEY SAMPLES

All the tested honeys were stored in dark at room temperature until used. The honey solution was handled aseptically. Fifty gram of each honey was weighed and mixed into a 100 ml of sterile distilled water to achieve 50% (w/v) solution, and the solution was further diluted in sterile distilled water to achieve solutions containing 25% (v/v), 20% (v/v), 15% (v/v) and 10% (v/v). These solutions were used to saturate paper disks in assays in order to determine zones of inhibition.

To evaluate the osmotic pressure's effect, artificial honey was used. A sugar solution [80% (w/v) sugar], served as an artificial honey control, was prepared by dissolving 40 g of fructose, 30 g of glucose, 8 g of maltose, and 2 g of sucrose in 100 ml of sterile distilled water and diluted till 50% (w/v), 25 (w/v) and 20 (w/v).

4.1.4 ASSESSMENT OF ANTIBACTERIAL ACTIVITY

Antibacterial activity of honey was analyzed using a disc diffusion assay according to the technique

describe by Bauer *et al.* (1966) with some adaptation by Taormina *et al.* (2001), by using a sterile paper disks (Whatman - type 3) 5 mm of diameter.

4.1.5 PRELIMINARY SCREENING OF SUSCEPTIBILITY OF BACTERIA TO HONEY

Preliminary screening was carried out only with the concentration of 50 % (w/v). Petri dishes of 12x90 mm were prepared with 9 disks, 6 of the disks were impregnated with 5 mg of honey in 10µl of honey solution at the concentration of 50 % (w/v) and of the other 3 disks, the first one was impregnated with 1µg of penicillin G (Oxoid Microbiology Products, UK) and the second with sugar solution described in the section 4.1.4 and the third with water and were used as positive (the first) and negative controls (the second and the third).

Using a loop 3-5 identical colonies were picked from a blood agar plate (Biotrading) culture and transferred into a tube containing 5 ml of Brain Heart Infusion (BHI) broth (Becton Dickinson). The broth culture was incubated under anaerobic conditions at 37°C until it achieved or exceeded the turbidity of 0.5 McFarland standard (a 0.5 McFarland standard was prepared by mixing 0.05 mL of 1% $BaCl_2 \cdot 2H_2O$ with 9.95 mL of 1% H_2SO_4). The incubation time was 2-6 hours. After adjusting the turbidity of inoculums suspensions, a sterile cotton swab was dipped into the adjusted suspension, and streaked over the entire sterile BHI agar surface. Then antimicrobial disks with 10µl of 50% (w/v) of honey solutions, positive control and negative controls were dispensed onto the surface of inoculated agar plate.

After incubation time at 37°C for 24 hours under anaerobic conditions, zones of inhibition surrounding the discs were measured with a ruler and the average of diameter of zones were recorded.

4.1.6 DETERMINATION OF MINIMAL INHIBITORY CONCENTRATION (MIC) OF HONEYS

Honeys which presented zone of inhibition > 6 mm of diameter were selected for MIC analysis by using agar disc diffusion. In this step the same technique as described in the preliminary screening was used with the exception that paper discs were impregnated with 20µl of honey solution instead of 10 µl. The tested concentrations were 50%, 25%, 20%, 15%, 10% corresponding disks containing 10 mg, 5 mg, 4 mg, 3 mg and 2 mg of the honeys, respectively. Determined MICs corresponds to the lowest concentration, for which a zone of inhibition was visually detectable.

4.2 RESULTS

4.2.1 PRELIMINARY SCREENING OF SUSCEPTIBILITY OF BACTERIA TO HONEY

In the preliminary screening all the honeys types were tested against *C. perfringens* type A strain. The concentrations of the honey solutions were 50% (w/v). The activity of the tested honeys was expressed by zone of inhibition. Results are shown in table 2.

Table 2. Growth inhibition expressed as zone of inhibition.

Sample	Zone of inhibition (diameter in mm) mean ± SD)
A	5 ± 0
B	8.3 ± 2
C	7.5 ± 0.7
D	5 ± 0
E	11 ± 2
F	14.3 ± 0.6
SS	6.1 ±1.5
P	30.8 ± 1.4
N	5.2 ± 0.5

SS = Sugar solution; P = positive control; N = negative control (H_2O)

From all the tested honeys Finnish organic honeys B, C, E and F showed antibacterial activity compared to water control (diameter of zone of inhibition > 5.2 ± 0.5 mm). All these four honeys showed better antibacterial activity than control artificial honey sugar solution (diameter of zone of inhibition 6.1 ±1.5 mm). The broadest zone of inhibition against *C. perfringens* was induced by honey F (diameter of zone inhibition 14.3 ± 0.6 mm) followed by honey E (diameter of zone of inhibition 11 ± 2 mm) and honey B with the zone of inhibition diameter of 8.3 ± 2 mm and honey C with zone of inhibition diameter of 7.5 ± 0.7 mm; honeys A and D did not show any antibacterial activity against *C perfringens*. Negative water control did not show activity and positive antibiotic control was constantly high (diameter of zone of inhibition of 30.8 ± 1.4 mm).

4.2.2 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

After preliminary screening of the organic honeys against *C. perfringens* strain, honeys that have shown antibacterial activity higher than artifisial honey control (zone of inhibition 6.1 ±1.5) were tested at different concentrations of 50%, 25%, 20%, 15%, and 10% corresponding disks containing 10 mg, 5 mg, 4 mg, 3 mg and 2 mg of the honeys, respectively, in order to determine the MICs. MIC corresponds to the lowest concentration, for which an inhibition zone was visually detectable. The effectiveness of antibacterial activity of the honeys was based on Minimum inhibitory concentration values. The results of the determination of the MICs of the honeys against the isolate of *C. perfringens* are shown in Table 3.

Table 3. Determination of the Minimum inhibitory concentration (mg) of honey samples (A, C, E, F) and sugar solution against *C. perfringens*

Sample	mg 50%	5mg 25%	4mg 20%	3mg 15%	2mg 10%
B	+++	+++	+++	++	+
C	+++	+++	++	++	+
E	+++	+++	+++	++	-
F	+++	+++	+++	++	++
SS	++	+++	++	NA	NA

+++ = Good inhibition (> 8mm);
++ = Moderate inhibition (7-8 mm);
+ = weak inhibition (6-7 mm);
SS = Sugar solution;
- = No inhibition (0-6 mm);
NA = Not analyzed

The results in the table 3 show that the better antibacterial activities of the studied honeys than induced by artificial honey, sugar solution SS, were achieved mainly by using high honey concentrations. All the tested honeys except C showed good and clear antibacterial activity against *C. perfringens* even at the concentration of 20 % (4 mg) compared to the control sugar solution. At the honey concentrations of 15% (3 mg) and 10% (2 mg) the zone of inhibitions around the discs were not clear. The most efficient honey against *C. perfringens* *was* honey F that showed moderate antibacterial activity even at the concentration 10% (2 mg).

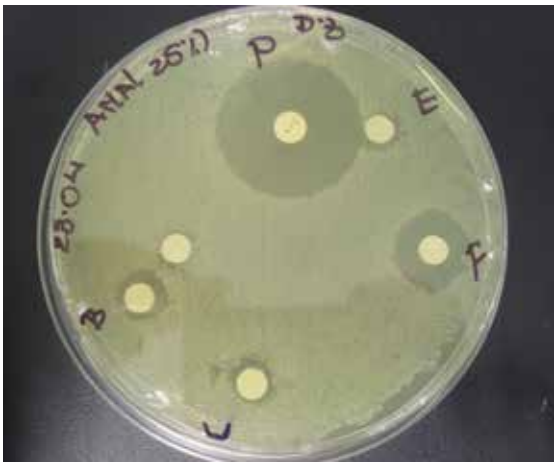

Figure 2. The zone of inhibition against *C. perfringens*. Honeys B, C, E and F at the concentration of 25 % (5 mg) was studied; P = positive control, no mark = sugar solution

4.3 DISCUSSION

Because of increasing drug resistance also against *C. perfringens* strains new antimicrobials are needed. Antibacterial activity of honey has been investigated for its potential use in reducing foodborne pathogens (Taormina *et al.,* 2001). Our data show that from the six organic honeys tested four had antibacterial action and two of the honeys had no activity against *C. perfringens* strain. This is the first report on the antimicrobial activity of organic honeys against *C. perfringens* and antibacterial activity of organic honeys.

There are many factors in honey that effect on the growth of *C. perfringens*. *C. perfringens* is not especially tolerant of low water activity (a_w), and reported values are between 0.93-0.97 for *C. perfringens* (Labbe, 1989). Honey is a supersaturated sugar solution with 0.56-0.62 a_w (Molan, 1992), which partly explains the inhibitory activity against *C. perfringens*. In our study, a sugar control, artificial honey, was used to eliminate the hyperosmotic effect of honey in the results. Antimicrobial activity was recorded, when the zone of inhibition was higher than induced by the sugar control.

Like most microorganisms, *C. perfringens* initiate growth most readily under neutral pH conditions, although excellent growth occurs at values between pH 6 and 7. Smith (1972) reported that the growth of *C. perfringens* is severely limited at pH ≤ 5,0 and 8, 3≥. The pH of honey is between

3.2 and 4.5. All the active honeys showed activity in dilutions up to 15% (at the concentration of 3 mg), which raises pH of the honey sample.

In 1984 Kokubo *et al.* found spores of Bacillus and Clostridium in honey from processing plants and retailers, when 56 of 71 of the studied samples contained clostridial spores, with 6 samples containing *C. perfringens.* Taormina *et al.* (2001) reported that the growth of *B. cereus* strains were not inhibited by studied honeys and thus *B. cereus* may reflect a generally higher tolerance to antimicrobials. This higher tolerance of *C. perfringens* was not seen in our study, where *C. perfringens* was inhibited by the four of the six studied honeys, which were certified organic honeys. Organic honeys have not been investigated before as regards their antibacterial activity and may have unknown antimicrobial factors.

In the present study four of the studied five Finnish organic honeys B, C, E and F had antimicrobial activity. Honey D from Central Finland was the only one of the five tested Finnish honeys that did not show antimicrobial activity. It did not have crystals and had been treated by heating at 50°C, all the other honeys had crystals and were untreated. Honey A from Argentina and Hungary was untreated and it had crystals, but it did not show inhibitory activity.

The most efficient honey against C. perfringens was honey F that showed moderate antibacterial activity even at the concentration of (10%). Its additional antimicrobial activity may be due to other factors than pH or hyperosmotic effect. The variation on antibacterial activity of the honeys could be attributed to the floral source or geographic region from which the honey was produced. In honey F the main floral source was willow herb. In our study Huttunen *et al.* (2013) with Finnish monofloral honeys we reported that the best antibacterial activities were received with willow herb (*Epilobium angustifolium*) and heather (*Calluna vulgaris*) and buckwheat (*Fagopyrum esculentum*) honeys against the studied human streptococcal and staphylococcal pathogenic bacteria. We also reported that willow herb, heather and buckwheat honeys were active against bacteria after heating indicating the presence of other antimicrobial component than hydrogen peroxide. In the present work the effect of hydrogen peroxide cannot be excluded, because no heat treatment or catalase addition was included in the study.

In honey E the main floral source was clover. Honey E had the second best activity after honey F. Clover honey has been reported to possess antimicrobial activity against *Pseudomonas aeruginosa* (Lu *et al .,* 2013). The main floral sources in honeys B and C were wild raspberry, willow herb, lingonberry and bilberry and the antibacterial activity was quite similar in honeys B and C. In honey D, which was negative, the main floral sources were wild raspberry and lingonberry. The difference compared to other studied honeys was that honey D was treated by heating it shortly to 50°C. One may speculate that heating have destroyed the active component, which thus suggests to be hydrogen peroxide. In the present work the active components in the studied organic honeys remain open and further studies will be needed.

In conclusion, we here report for the first time antimicrobial activity of Finnish organic honeys against *C. perfringens*, a food poisoning bacterium. This is also the first report on antibacterial activity of organic honeys, and antibacterial activity of honey against *C. perfringens.* As shown in previous studies (Huttunen *et al.,* 2013) honeys have varying and diverse effects on the growth of each bacterial strain and each organism have unique response profile to different honeys. This gives rationale for further studies on antimicrobial activity of organic honeys against food poisoning bacteria including characterization of the antimicrobial components. Considering honey as preservative in food products especially in organic food it would be important to characterize the antimicrobial components and evaluate the effect of heat treatments on antimicrobial activity.

5 SINGLE PCR FOR NETB TOXIN TYPING

5.1 MATERIAL AND METHODS

5.1.1 BACTERIAL ISOLATES AND CULTURING METHOD

From a previously described collection of 65 *C. perfringens* isolates from broilers (Nauerby *et al.,* 2003), 43 isolates have been received from the National Food Institute, Søborg Denmark and were used in this study. The *C. perfringens* positive and negative control strains for netB toxin typing were kindly provided by Leen Timbermont (University of Ghent, Belgium). From producers 1, 2 and 3, the isolates were sampled from presumably healthy birds. From producers 4, 5, 6, 7, 9, 15, 17, 20, 21, 22, 25, 26 and 27 the isolates were collected from several chickens suffering from severe NE.

The isolates were cultured on blood agar containing 5% defibrinated sheep blood and incubated anaerobically (AnaerobicCult A, Merck, Darmstadt, Germany) at 37°C for 24 to 48 h.

5.1.2 DETECTION OF THE NETB TOXIN

DNA extraction of C. perfringens isolates
Extraction of DNA from all *C. perfringens* isolates was performed using a boiling technique. Briefly, few colonies of bacteria grown on a blood agar plate were suspended in 2 ml sterile water to a suspension of McFarland No. 0.8 – 1.2. After the measurement, 500 µl of the suspension were transferred into an Eppendorf tube and was boiled for 10 min at 100 °C. The DNA preparations were stored at -20 °C until use.

Single PCR for NetB toxin typing
NetB toxin was detected by the PCR method described by Keyburn et al. (2008) with a few modifications. The PCR amplification was performed in 28 µl volumes containing 5× Phire Reaction Buffer (Finnzymes), 10 mM dNTP mixture (Finnzymes), 1 U of Phire Hot Start DNA polymerase (Finnzymes), 10 pM of primer (AKP78 5` GCTGGTGCTGGAATAAATGC-3` and AKP79 5`TCGCCATTGAGTAGTTTCCC-3`), 14.5 µl of distilled water and 5 µl of template. The primers were synthesized by Oligomer Oy. The PCR was performed in a MiniOpticon™ System cycler and the conditions were one cycle of 94 °C for 1 min followed by 35 cycles at 94 °C for 15 sec; 55 °C for 15 sec, 72 °C for 30 sec, with the final step at 72 °C for 12 min. The PCR product was loaded onto a 1.5 % agarose gel (1.35 % SeaKem® LE Agarose and 0.65 % NuSieve® GTG Agarose, Cambrex Bio Science, Rockland, ME, USA) containing 0.1 g ml^{-1} ethidium bromide. A DNA molecular weight marker 100 bp low ladder (Sigma-Aldrich, Saint Louis, MO, USA) was included in each gel. The gel was photographed under UV light (Alpha DigiDoc, Alpha Innotech, San Leandro, CA, USA). The PCR reaction for each sample was performed three times and considered positive if the primer set gave a distinct band of the right size (383bp).

5.2 RESULTS

All isolates included in the present study were previously found to be of Type A (Nauerby *et al.*, 2003). Out of all 43 isolates, the *netB* gene was detected in 13 (30%) isolates from broilers suffering from NE and in 14 (32.5%) isolates from healthy broilers.

5.3 DISCUSSION

The *C. perfringens* necrotic enteritis toxin B(NetB) was recently proposed as a new key virulence factor for the development of NE in broilers (Keyburn *et al.*, 2008, 2010). In the present study, the *netB* gene was detected in 30% *C. perfringens* isolates from broilers suffering from NE and in 32.5% isolates from healthy broilers. Martin and Smyth (2009) found the *netB* gene in 14 isolates from studied 92 chickens 7 from chickens with necrotic enteritis, and 7 from unrelated chickens with no evidence of necrotic enteritis. Keyburn et al. (2010) reported that 70 % (31/44) of the strains isolated from NE-affected birds were *netB*-positive, whereas only two out of 55 isolates from healthy chickens carried the netB gene. Analysis of strains from various Danish

broiler flocks showed that 52% and 61% of isolates from birds recovered from NE, and from healthy birds, respectively, were *netB* positive (Abildgaard *et al*., 2010). study of Norwegian broilers cholangiohepatitis Chalmers *et al*. (2008) described that 95 % (39/41) of all *C. perfringens* isolates from broiler with NE carried netB and 35 % (7/20) from healthy broiler. These results imply that although there is a clear association between the presence of *netB* toxin gene and development of NE there may be other virulence factors that are produced by these *netB*-negative disease-producing isolates. The NetB toxin may not be an obligate requirement for poultry *C. perfringens* virulence and at least the presence of *netB* may not be essential for the disease process in all *C. perfringens* isolates. The role of NetB in the introduction of NE needs further investigations.

REFERENCES

Abildgaard L, Sonderaard T, Engeberg R, Schramm A, Hojberg O. *In vitro* production of necrotic enteritis toxin B, by *netB*-positive and *netB*-negative *Clostridium perfringens* originating from healthy and diseased broiler chickens. Vet Microbiol 2010; 144:231-235.

Al-Sheikhly F, Truscott R. The interaction of *Clostridium perfringens* and its toxins in the production of necrotic enteritis of chickens. Avian Dis 1977; 21:256-263.

Bauer AW, Kirby WM, Sherris JC, Turk M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 1966; 45:493-496.

Baums CG Schotte U, Amtsberg G and Goethe R. Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. Vet Microbiol 2004; 100, 11-16.

Branikl FJ. Surgery in Western Kenya. Ann R Con Surg Eng. 1981; 63:348-352.

Carman RJ, Perelle S, Popoff MR. 1997. In Rood JI, McClane BA, Songer JG Titball RW (edit). The clostridia: molecular biology and pathogenesis. Academic Press, San Diego, California, pp. 359-369.

Chalmers G, Bruce HL, Hunter BD, Parreira VR, Kulkarni RR. Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. J Clin Microbiol 2008; 46:3957-3964.

Chen JC, Lamikanra O, Banks D, Hunter PA. Biochemical and microbial changes during the storage of minimally processed cantaloupe. J Agric Food Chem 2000; 48:5955-5961.

Cooper RA, Molan PC, Harding KG. The effectiveness of the antibacterial activity of honey against strains of *Staphylococcus aureus* isolated from infected wounds. R Soc Med 1999; 92: 283-285.

Crane E. 1999. History of use of honey. In The world history of beekeeping and honey hunting Gerald Duckworth and Company Ltd. London, pp. 502-511.

Davidson PM. 2001, Chemical preservatives and naturally antimicrobial compounds. In Doyle MP, Beuchat LR, Montville TJ (edit) Food Microbiology. Fundamentals and frontiers, ASM Press, Washington, DC, pp. 593–628.

Daube G, Simon P, Limbourg B, Manteca C, Mainil J, Kaeckenbeeck A. Hybridization of 2,659 *Clostridium perfringens* isolates with genes probes for seven toxins (alpha, beta, epsilon, Iota, teta, and enterotoxin) and for sialidase. Am J Vet Res 1996; 57:496-501.

Dold D, Du DH, Dziao ST. The antibacterial, heat and light sensitive inhibitory substance, inhibine, in natural honey. Z Hyg Infektionskrankh 1937; 121:333-333.

Duffy G, Walsh C. 2005, Antibiotic Resistance in foodborne pathogens. The National Food Centre, Auston, Dublin 15, pp.10-11.

Estevinho L, Pereira AP, Moreira L, Pereira E. Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. Food Chem Toxicol 2008; 46:3774-3779.

Fujiwara S, Imai J, Fujiwara M, Yaeshima T, Kawashima T, Kobayashi K. A potent antibacterial protein in royal jelly. J Biol Chem 1990; 265: 11333-11337.

Gould GW. Ecosystem approaches to food preservation. Soc Appl Bacteriol Symp Ser 2159S-68S.

Huang H. 2007, *Clostridium perfringens* Sporulation and gliding motility. Dissertation. Oregon State University, Oregon, pp. 2-10.

Huttunen S, Riihinen K, Kauhanen J, Tikkanen-Kaukanen C. Antimicrobial activity of different Finnish monofloral honeys against human pathogenic bacteria. APMIS 2013;121:827-834.

James MJ 2005 Modern food microbiology. Aspen Publishers, Inc. 6th ed.Gaithersburg, Maryland, pp.461-466.

Kokubo Y, Jinbo K, Kaneko S, Matsumoto M. Prevalence of spore-forming bacteria in commercial honey. Ann Rep Tokyo Metr Res Lab Pub Health 1984; 35:192-196.

Keyburn AL, Sheedy LSA, Ford ME, Williamson MM, Awad MM, Rood JI, Moore RJ. Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. Infect Immun 2006; 74:6496-6500.

Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Rubbo DIA, Rood A JI, Moore R.J. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. Plos Pathogens 2008; 4:26-26

Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Rubbo DIA, Rood A JI, Moore R.J. NetB, a Pore-Forming Toxin from Necrotic Enteritis Strains of *Clostridium perfringens*. Toxins 2010; 2:1913-1927.

Kwakman PHS, te Velde AA, de Boer L, Speijer D, Vandenbroucke-Grauls CMJE, Zaat, SAJ. How honey kills bacteria. FASEB J 2010; 24: 2567-2582.

Kwakman PHS, te Velde AA, de Boer L, Vandenbroucke-Grauls CMJE, Zaat, SAJ. Two Major Medicinal Honeys Have Different Mechanisms of Bactericidal Activity. PLoS One 2011, 6,e17709.

Kwakman PHS, van der Akker JPC, Güclü A, Aslami H, Binnekade JM, de Boer L, et al. Medical grade honey kills antibiotic-resistant bacteria in vitro and eradicates skin colonization. Clin Infect Dis 2008;46:1677-1682.

Labbe RG. 1989. *Clostridium perfringens*, In Doyle MP (edit) Foodborne bacterial pathogens. Marcel Deker, Inc, New York, pp.192-234.

Labbe RG, Juneja VK. 2006*, Clostridium perfringens* gastroenteritis. In Riemann HP and Cliver DO (edit). Foodborne infections and intoxications Elsevier Academic Press, the Netherlands, pp.138-164.

Lu J, Carter DA, Turnbull L, Rosendale D, Hedderley D, Stephens J, Gannabathula S, Steinhorn G, Schlothauer RC, Whitchurch CB, Harry EJ. 2013. The effect of New Zealand kanuka, manuka and clover honeys on bacterial growth dynamics and cellular morphology varies according to the species. PLoSOne. 2013; 8: e55898. doi: 10.1371/journal .pone. 0055898. Epub 2013 Feb 13.

Martin TG, Smyth JA. Prevalence of *netB* among some clinical isolates of *Clostridium perfringens* from animals in the United States. Vet Microbiol. 2009;14:136:202-5. doi: 10.1016/j. vetmic.2008.10.026. Epub 2008 Oct 31.

Mavric E, Wittmann S, Barth G, Henle T. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of manuka (*Leptospermum scoparium*) honeys from New Zealand. Mol Nutr Food Res 2008; 52: 483-489.

McKibben J, Engeseth NJ. Honey as a protective agent against lipid oxidation in ground Turkey. J Agric Food Chem 2002; 50:592-595.

Melissa A, Olga I, Zakour P, Worobo RW. Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. Int J Food Microbiol 2004; 97:1-8.

Molan PC. The antibacterial activity of honey. 1. The nature of antibacterial activity. Bee World 1992; 73:5-28.

Molan PC. Why honey is effective as a medicine. 2. The scientific explanation of its effects. Bee World 2001; 82:22-40.

Nauerby B, Pedersen K, Madsen M. Analysis by pulsed-field gel electrophoresis of the genetic diversity among *Clostridium perfringens* isolates from chickens. Vet Microbiol 2003; 94:257-266.

Petit L, Gibert M, Popoff MR. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol 1999; 7:104-110.

Smith L. Factors involved in the isolation of *Clostridium perfringens*. J. Milk Food Technol 1972; 35:71-76.

Sterne M, Warrack GH. The types of *Clostridium perfringens*. J Pathol Bacteriol 1964; 88: 279-283.

Taormina PJ, Niemira BA, Beuchat LR. Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. Int Jour Food Microbiol 2001; 69:217–225.

Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol. 2011: 40341-40347. doi:10.1080/0307 9457.2011.590967.

Teuber M. Spread of antibiotic resistance with food-borne pathogens. CMLS Cell Mol Life Sci 1999; 56:755-763.

Tonks A, Cooper RA, Price AJ. Stimulation of TNF-a releas in monocytes by honey. Cytokine 2001; 14:240-242.

Wen Q, McClane BA. Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. Appl Environ Microbiol 2004; 70:2685–2691.

White JW, Subers MH, Schepartz A. The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. Biochim Biophys Acta 1963; 73:57–70.

Zumla A, Lulat A. Honey - a remedy rediscovered. J Royal Soc Med 1989; 82:384-385.

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