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题　　目 丁香酚和芸香苷对副溶血弧菌致病性的影响

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学生姓名 段菁岳

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指导教师 孙群；Kumar Venkitanarayanan

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段菁岳

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Effect of eugenol and rutin hydrate on *Vibrio parahaemolyticus* virulence

Jingyue Duan

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学生姓名 段菁岳　指导老师 孙群, Kumar Venkitanarayanan

摘要

副溶血弧菌是一种主要存在于海水及海产品中的嗜盐革兰氏阴性病原菌，食用带有该菌的食物或者未熟的海产品可致食物中毒以及急性肠胃炎。临床上以急性起病、腹痛、头痛、呕吐、腹泻及水样便为主要症状。副溶血弧菌的主要致病因子包括运动能力，对宿主小肠上皮细胞的粘附能力，以及耐热直接血溶素的产生。减少上述致病因子的产生可以用来控制副溶血弧菌在人类中的感染。丁香酚和芸香苷是分别从植物丁香和槐米中提取出精油的主要成分，它们有抗菌及抗氧化的功效。本毕业设计研究了两种植物提取抗菌素丁香酚和芸香苷在半抑制浓度（该浓度是抗菌剂在不抑制细菌的生长，能够对细菌的外部结构进行修饰的同时，抑制其致病因子如运动能力、粘附能力、表面敏感性以及致病蛋白表达等所需的最高浓度）下，在体外对副溶血弧菌的关键致病因子表达量的减少。所有的实验在两株副溶血弧菌（RIMD2210633，ATCC17802）上进行，并有重复样本，实验重复三次。基于在不同浓度抑菌剂以及对照组下细菌的生长状态，丁香酚和芸香苷的半抑制浓度分别确定为 0.03%以及 0.07%。与对照组比较，实验组丁香酚和芸香苷分别减少了副溶血弧菌 44%和 73%的运动能力。另外，通过细胞实验可知，丁香酚和芸香苷分别减少了副溶血弧菌 44%和 73%的粘附能力。实验结果表明，丁香酚和芸香苷可以被用来减少副溶血弧菌的致病力，然而，在应用这些结果之前，模式动物及体内实验的验证是必须的。

关键词：副溶血弧菌、丁香酚、芸香苷、半抑制浓度

Effect of Eugenol and Rutin hydrate on *Vibrio parahaemolyticus* virulence

Major: Biology

Student: Jingyue Duan

Advisor: Dr. Qun Sun (SCU), Dr. Kumar Venkitanarayanan (UCONN)

Abstract

Vibrio parahaemolyticus is a foodborne pathogen which causes an acute gastroenteritis associated with the consumption of contaminated raw or under cooked seafood. The major virulence factors of *V. parahaemolyticus* include motility, adhesion to host intestinal epithelium, and thermostable direct hemolysin (TDH) production. Reducing the production of these virulence factors could control *V. parahaemolyticus* infections in humans. This study investigated the efficacy of sub-inhibitory concentrations (SICs; compound concentrations not inhibiting bacterial growth) of two plant-derived antimicrobials, namely Eugenol (EG) and Rutin hydrate (RH) in reducing the expression of critical virulence factors of *V. parahaemolyticus in vitro*. All experiments were conducted on two strains of *V. parahaemolyticus* (RIMD2210633, ATCC17802) with duplicate samples, and the study was replicated three times. Based on bacterial growth, the SIC of EG and RH were 0.03% and 0.07%, respectively. Eugemol and RH reduced *V. parahaemolyticus* motility by 44% and 73%, respectively compared to control. In addition, cell culture assay revealed that both EG and RH reduced *V. parahaemolyticus* adhesion to Caco-2 cells by 15% and 85%, respectively. Results suggest that EG and RH could potentially be used to reduce *V. parahaemolyticus* virulence, however, *in vivo* studies are necessary to validate these results.

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I. INTRODUCTION

Vibrio parahaemolyticus is a Gram negative, motile, facultative anaerobic, halophilic food-borne pathogen widely distributed in the marine fauna and marine water [1]. *V. parahaemolyticus* was first recognized as a seafood-borne pathogen in 1950 in Japan [1] and since then several sporadic outbreaks have been reported throughout the world, especially in Asia [2]. During 1991 to 2001, *V. parahaemolyticus* caused 31.1% of 5770 food-borne outbreaks reported in China [3, 4], and it was the major cause of food poisoning (1710 incidents, 24,373 cases) in Japan between 1996 and 1998 [5]. In addition, *V. parahaemolyticus* is the major cause of human gastroenteritis associated with seafood consumption in the United States [5]. Between 1973 and 1998, approximately 40 outbreaks of *V. parahaemolyticus* infections were reported according to the Centers for Disease Control and Prevention [6]. Although *V. parahaemolyticus* primarily causes acute gastroenteritis with rapid onset of symptoms [2], it also can cause wound infections and septicemia [6]. Infected individuals would develop severe dehydration, and cyanosis [7]. Infections could also lead to cardiovascular abnormalities [8], and autopsies have revealed extensive damage to the stomach, other components of the gastrointestinal tract, and internal organs [9]. The major food associated with *V. parahaemolyticus* outbreaks include raw or undercooked seafood [10].

A number of *V. paraheamolyticus* virulence factors critical for its colonization and infection have been identified. These include adhesins and flagella [11] and hemolysins [12] such as thermostable direct hemolysin (TDH). Bacterial motility is essential for host-microbial interactions, colonization and virulence in the host [13]. Motility helps *V. parahaemolyticus* for traversing through intestine to reach a favorable niche. Moreover *V. parahaemolyticus* adhesion to the host intestinal cells is a critical step in initiating a successful infection in the host. TDH is another major virulence factor of *V. parahaemolyticus* and is responsible diarrhea [10]. Thus, reducing *V. parahaemolyticus* motility, attachment to host intestinal tissue as well as production of TDH could potentially control *V. parahaemolyticus* related acute gastroenteritis in humans.

Although antibiotics are the drug of choice for treating *V. parahaemolyticus* infection, an increasing resistance to antibiotics in the bacterium has been reported[14], thereby emphasizing the need for alternate strategies for controlling the pathogen. The use of natural plant essential oils as antimicrobial agents have received significant attention in the past decade due to concerns over development of antibacterial resistance in pathogens [15, 16]. Traditionally, plant-derived antimicrobials (PDAs) have been used as flavor enhancers, and preservatives in many ancient cultures. In addition, they have been used for treating various ailments and diseases in traditional Chinese and Indian medicine [17]. A plethora of plant-derived compounds with significant antimicrobial properties have been identified [16]. Eugenol (EG) is a natural ingredient present in the essential oil extracted from cloves [18], and a Generally Recognized as Safe (GRAS) compound approved for use in foods (19 CFR 184.1257). Rutin hydrate (RH) is a major flavonoid derived from Fagopyrum esculentum [19] and possesses antimicrobial [20] and antioxidant [21] properties.

The antimicrobial efficacy of EG against *Salmonella* Typhi [18], *Listeria monocytogenes* [22], *Escherichia coli* O157:H7 [23], *Psuedomonas aeruginosa* and [24] that of RH against *E. coli*, *E. faecalis* and *Proteus vulgaris* [25] has been reported earlier. However, no studies determining the effect of EG and RH on *V. parahaemolyticus* virulence factors have been conducted.

The objective of this study was to investigate the effect of sub-inhibitory concentration (SIC, concentration not inhibiting bacterial growth) of EG and RH on the virulence factors of *V. parahaemolyticus.*

II. LITERATURE REVIEW

1. History

Vibrio parahaemolyticus was first isolated in 1950 from an outbreak in Japan associated with ingestion of partially boiled sardines, which resulted in 272 human illnesses and 20 deaths [6]. Since then, *V. parahaemolyticus* has been recognized as a common marine pathogen causing seafood-borne illness in Japan and throughout the world [5]. Many researchers have studied the distribution of *V. parahaemolyticus*. It is generally accepted that the occurrence of outbreak is highest in estuarine or coastal areas of the world oceans [26], and the outbreaks show the same seasonal pattern [27]. In the United States, the first strain of *V. parahaemolyticus* was isolated from the marine environment of Puget Sound and inshore coastal in 1970 [28]. Subsequently, *V. parahaemolyticus* was recognized as a foodborne pathogen in the first reported case that occurred in Maryland due to consumption of undercooked crabs [29]. *V. parahaemolyticus* was first identified through DNA-DNA hybridizations studies conducted by Nishibuchi in 1985 [30].

2. Biology

Vibrio spp. are Gram negative, halophilic, facultative anaerobic [31], straight or curved motile organisms. All the species within the genus *Vibrio* carry two chromosomes with some species carrying additional large plasmids [32]. *Vibrio spp.* can typically grow at 35℃ on thiosulfate-citrate-bile salts-sucrose (TCBS) agar, and 1% NaCl is essential for culturing Halophilic *Vibrio* [33]. Isolation of *V. parahaemolyticus* from *Vibrio spp.* on TCBS agar has been found ineffective [34]. The green colonies of *V. parahaemolyticus* on TCBS are undistinguished with *V. mimicus and* and *V. vulnificus* [35], or be covered by a yellow color produced by sucrose-fermenting bacteria *V. cholerae* and *V. alginolyticus* [36]. DNA hybridization studies revealed that genes such as *toxR* [37], *tdh* and *trh* [38] are specific for *V. parahaemolyticus* and are used for rapid detection of *V. parahaemolyticus* strains in food samples or clinical specimens. *V. cholerae* [39], *V. parahaemolyticus* [40], *V. vulnificus* [41] and *V. alginolyticus* [42] are four well-documented human pathogens under the genus Vibrio. Infections by theses organisms are usually associated with

consumption of raw or undercooked seafood.

3. *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a Gram-negative, motile, facultative anaerobic, halophilic, bacterium. Depending on the environmental conditions, it exhibits swinning motility in liquid environment with a single polar flagellum , or a swarming motility in highly viscous environment by lateral flagella [13]. *V. parahaemolyticus* also have the ability to produce capsule to enhance its virulence which protect bacteria cells from engulfment by macrophages in the host [43]. *V. parahaemolyticus* strains are classified by serotyping, which depends on the combination of antigen types on the somatic (O) and capsular (K). Thirteen different O antigens [44] and 71 different K antigens have been identified in *V. parahaemolytics* [45]. The most common serotype of *V. parahaemolyticus* is O3:K6 since 1996 [46], after the first illness reported a Japanese traveler returning from Indonesia in 1995 [47]. A diarrhea outbreak occurred in India in 1996 with 50-80% of isolates belonging to O3:K6 [45]. Similarly, 83.8% of isolates associated with an outbreak in 1997 in Taiwan belonged to O3:K6 an [48].

4. Virulence factors of *Vibrio parahaemolyticus*

Virulence factors refer to the compounds produced by pathogens that enable them to replicate and disseminate within a host by destroying or evading host defenses [49], and enhance their potential to cause disease. The general virulence factors in bacteria include bacterial and exotoxins [10], outer membrane proteins that mediate bacterial attachment or invasion of enterocytes [50], and polar flagellum that facilitate motility in host gut [51].

The major virulence factors of *V. parahaemolyticus* include adhesin, vibrioferrin [46], thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) [52], and type III secretion systems (T3SS1 and T3SS2) [53]. Most strains isolated from the environment or seafood are not hemolytic or non-pathogenic [54], whereas strains of *V. parahaemolyticus* isolated from human fecal samples (including food-borne disease cases and healthy carriers) are pathogenic, exhibiting hemolysis on blood agar [55].

4.1 Flagella and motility

V. parahaemolyticus has a single polar flagellum powered by the sodium motive force, and is required for bacterial motility [13]. On semisolid surfaces or highly viscous environment, flagella switches into the proton-powered lateral flagella-driven swarm motility [46]. The flagellar systems conribute to *V. parahaemolyticus* colonization of the human host [56].

4.2 Adhesin

Adhesins are bacterial surface proteins involved in initial contact and anchoring to a host cell [52], and play an essential role for the activation and delivery of latter secreted effectors and toxins [46]. *V. parahaemolyticus* contains an outer membrane protein that mediates initial attachment to host cells [52]. The mammalian cell entry (mce) adhesion molecule (MAM7) was identified to be essential for initial contact of *V. parahaemolyticus* with cultured host cell lines, and plays a role in *V. parahaemolyticus* pathogenesis [46, 57]. MAM7 interacts with host cell surface protein, fibronectin and phospholipid, phosphatidic acid on plasma membrane, and that three molecules result in a tripartite complex on the bacterial and host cell surface [52, 58].

4.3. Vibrioferrin

Iron is an essential element for most microorganisms, and the ability of these microbes to utilize iron is important for infection as well as survival in the environment [59]. Iron is used as the key component of many enzymes central to cellular processes such as electron transport, activation of oxygen, amino acid synthesis, DNA synthesis and peroxide reduction [60]. In humans, iron is bound by a number of different complexes, including transferrin, lactoferrin, and hemoglobin [61]. In order to establish an infection, bacteria have to adapt a system to utilize iron from these host-complexes [46]. The iron chelators in bacteria, siderophores, which are low molecular weight proteins that have high affinity for iron-binding component and transport iron back into the cell cytosol in the form of ferric-siderophore complex [62]. Siderphores produced by *V. parahaemolyticus* are known as vibrioferrin [63]. The subsequent transport of the ferric-vibrioferrin complex which combines with the outer membrane receptor protein encoded by *pvuA* gene [64] help *V. parahaemolyticus* to

compete for and acquire iron under the iron-limiting conditions [59].

4.4. Toxins

Almost all *V. parahaemolyticus* strains from clinical specimens possess β-hemolytic activity attributed to TDH or TDH-related hemolysin. These proteins were the first studied *V. parahaemolyticus* virulence factors identified in 1980s [65]. Those strains producing TDH are able to lysis human erythrocytes when plated on a high-salt media called Wagatsuma agar, which has been called the Kanagawa phenomenon (KP) [12]. The KP reaction is commonly used as a marker test for virulent strains, and only 1-2% of strains from nonclinical sources are KP positive [40]. However, some KP negative strains of V. parahaemolyticus cause gastroenteritis, however, these strains TRH rather than TDH [66]. Only a few of clinical strains contain both *tdh* and *trh* genes, and most of them possesses neither hemolysin genes [67].

4.4.1. Thermostable direct hemolysin

Thermostable direct hemolysin (TDH) is inactivated by heating at ~60-70 ℃, but is reactivated by additional heating above 80 ℃, this paradoxical phenomenon, known as the Arrhenius effect [68], which is tightly related to the reversibility of amyloid fibril formation upon heating of TDH, and its toxicity has been shown to associated with cholesterol and sphingolipid-enriched lipid [69]. The crystalline structure of TDH is a tetramer in solution with 50 Å in depth [70]. TDH has multiple biological activities, including hemolysis [71], enterotoxicity [72], cytotoxicity [73], and cardiotoxicity [74]. Thus, TDH has been considered a major virulence factor of *V. parahaemolyticus*.

4.4.2 TDH related hemolysin

TDH related hemolysin (TRH) has 67% similarity with TDH in amino acid level, and is tetramer in solution as well [75]. The molecular size of both purified TRH and purified TDH is both 23 kDa [76]. TRH also shares antigenicity in part with TDH and a high sequence homology (68%) exists between the *trh* and *tdh* genes [46]. TRH has less amyloid-like structure than TDH, but possesses a similar ability to lyse cells on red blood agar, although not identical [75]. In addition, unlike the Arrhenius effect of

TDH, TRH activity is inhibited upon heating at 60℃ or a higher temperature for 10 min because of incorrect refolding process [76].

4.5. The Type III secretion systems of *V. parahaemolyticus*

Type III secretion system (T3SSs) is a needle-like bacterial organelle that evolved to deliver bacterial secreted effectors and toxin protein directly into the cytoplasm of eukaryotic cells, and is encoded by mostly Gram-negative bacterial pathogens [77]. T3SSs show high similarities among different bacterial species, in delivering toxin effectors with the capacity to modulate a variety of cellular functions [78], including membrane disruption, cytoskeleton rearrangement, modulate intracellular trafficking and induction of apoptosis [79]. T3SSs consist of three main components: the basal body on bacteria membranes, a hollow needle-like structure [80] and the translocon, which works as a pore inserted into host cell [81]. They they function like a “bridge” between bacteria and eukaryotic cells, and transfer bacterial produced proteins without encountering the extracellular environment [82].

All *V. parahaemolyticus* strains encode T3SS1 on a pathogenicity island on chromosome 1, whereas T3SS2, encoded by chromosome 2, is only present in some clinical and environmental strains [53]. *V. parahaemolyticus* T3SS1 is related to its cytotoxic activity [83], while T3SS2 has been demonstrated to be involved in cytotoxicity and enterotoxicity [10]. *V. parahaemolyticus* T3SS1 causes cytotoxicity by producing effectors (VopQ, VopR, VopS, and VPA0450) into host cells such as macrophages and HeLa cells. T3SS2 effectors (VopA, VopC, VopL, and VopT) are translocated into host cells to cause cytotoxicity and enterotoxicity within the colon epithelial cells [46].

5. Plant-Derived Antimicrobial Compounds

Essential oils are plant-derived, natural, volatile compounds produced by certain aromatic plants as secondary metabolites [84]. They play an essential role in the protection of plants from virus, bacteria, fungus and insects [85]. In the food industry, plant-derived antimicrobials (PDAs) are traditionally used as flavoring or preserving agents in foods and beverages [86]. In the last decade, several researchers have investigated the efficacy of PDAs in controlling foodborne pathogens in foods due to

emergence of antibiotic resistance in pathogens and concerns over the use of synthetic antimicrobial compounds [87]. The antimicrobial properties of several plant-derived essential oils have been identified, which are proved to be effective and safe for use in food [16]. The current study utilized two such compounds with previously studied antimicrobial properties, eugenol (EG) and rutin hydrate (RH).

5.1 Eugenol

Eugenol (4-Allyl-2-methoxyphenol, $C_{10}H_{12}O_2$) is a phenylpropene, pale yellow oily aromatic liquid extracted from clove oil [88]. In medicine, eugenol is used as an analgesic, local anesthetic, anti-inflammatory [89], and antibacterial [90], where as in the food industry, eugenol is used as a food flavoring agentand antioxidant [91].

5.2 Rutin Hydrate

Rutin hydrate (Quercetin-3-rutinoside hydrate) is a polyphenolic flavonoid compound derived form buckwheat [19] that act as an antioxidant and scavengers of oxygen free radicals [92]. Rutin hydrate is used as an antibacterial, antioxidant and anti-inflammatory compound in medicine and has potential to control some varieties of cancers [93].

5.3 Antimicrobial effects of eugenol and rutin hydrate

The antimicrobial effects of these two plant-derived compounds have been previously reported. Eugenol inhibits the growth of *S.* Typhi [18], *L. monocytogenes* [22], *E. coli* O157:H7 [23], and *P. aeruginosa* [24], In addition, our laboratory had investigated the antibacterial effect of EG in inactivating *S.* Enteritidis [94] on eggs shell and *C. jejuni* in chicken cecal contents *in vitro* [95]. Other researchers have also shown that EG reduced coliform bacteria in the pig gut [96]. Similarly, previous studies investigating the antimicrobial effect of RH showed its ability to inhibit *E. coli, E. faecalis* and *P.vulgaris*, with MIC (minimum inhibitory concentration) values in the range of 2.5 mg/mL to 40 mg/mL [25].

6. Antimicrobial effect of plants derived antimicrobials on *V. parahaemolyticus*

Antimicrobial effect of various spices and herbs on *V. parahaemolyticus* has been demonstrated. It was observed that 0.5% of essential oils extracted from dried oregano or thyme were highly toxic to *V. parahaemolyticus* growth in growth media [97]. *V.*

parahaemolyticus was also sensitive to both basil and sage essential oils [98]. In addition, spices and herbs such asclove, garlic, and marjoramwere found to exert antibacterial activities at incubation temperatures of 30℃ and 5℃, whereas horseradish was antimicrobial on *V. parahaemolyticus* only at 30℃. The MIC of clove and marjoram against *V. parahaemolyticus* was 0.125% at 30℃ in a nutrient rich medium, while in a nutrient poor medium, the lowest MIC was 0.001 and 0.00025% at 30 ℃ and at 5 ℃, respectively [99]. Thus previous studies suggest that essential oils extracted from spices and herbs could be practical for protecting seafood from the risk of contamination by *V. parahaemolyticus* .

7. Hypothesis:

Based on published literature, it was hypothesized that EG and RH reduce *V. parahaemolyticus* virulence.

III. MATERIALS AND METHODS

1. Bacterial strains and media

Two strains of *V. parahaemolyticus* (RIMD2210633, ATCC17802) were used for this study. All bacteriological media used in the study were procured from Difco (Sparks, Md.). Each strain was cultured separately in 10 mL of sterile Luria Bertani broth with 10% NaCl in 15 mL centrifuge tubes at 37℃ for 24 h. The bacterial population in each culture was determined by serial 10-folds dilution in sterile phosphate-buffered saline (PBS) and plating 0.1 mL of diluted culture on duplicate Thiosulphate Citrate Bile Sucrose (TCBS) agar plates and incubated at 37℃ for 24 h.

2. Plant-derived compounds and SICs determination

The effect of SIC of EG and RH on *V. parahaemolyticus* was determined by culturing the pathogen either in the presence or absence of various concentrations of these plant compounds. The highest concentration of each plant compound that did not inhibit bacterial growth after 24 h of incubation at 37^{o}C was considered the SIC. Eugenol and rutin hydrate were purchased from Sigma. The SIC of each compound was determined by adding ~6.0 log/CFU of *V. parahaemolyticus* in 10 mL LB broth

with 1% NaCl, followed by the addition of 0%, 0.01%, 0.02%, 0.03% or 0.04% EG and 0%, 0.01%, 0.05%, 0.07% or 0.1% RH. Culture tubes were incubated at 37℃ for 24 h, and bacterial population was determined by serial dilution and plating on TCBS agar plates. Duplicate samples were included and the experiment was repeated three times.

3. Motility assay

The effect of SIC of EG and RH on *V. parahaemolyticus* motility was investigated using a standard motility assay (reference). Separate Luria Bertani (LB) agar (0.3%) plates containing the respective SIC of each plant compound were prepared. Agar not supplemented with the compounds served as control. An overnight cultured of *V. parahaemolyticus* was centrifuged at 3000 rpm for 30 min and resuspended in 200 μl phosphate buffered saline (PBS, pH 7.2). Twenty μl of resuspended culture (~ 8 log CFU/mL) was spot inoculated at the center of agar plates and incubated at 37℃ for 12 h, and the zone of motility was measured.

4. Cell culture

Human enterocyte-like Caco-2 cells (HTB-27) were obtained from the American type culture collection (Manassas, VA). Caco-2 cells were cultured in 25-cm2 tissue culture flasks (Falcon, Becton and Dickinson Company, Franklin Lakes, NJ) with minimum essential medium (MEM) (Gibco, Invitrogen, Carlsbad, CA) containing 10% (vol/vol) fetal bovine serum (Invitrogen). All cell cultures were incubated at 37℃ in a 5% (vol/vol) CO2 atmosphere.

5. Adhesion assay

The effect of SIC of EG and RH on *V. parahaemolyticus* adhesion to Caco-2 cells was investigated using a standard adhesion assay [100]. The Caco-2 cells were seeded in a 12-well tissue culture plates at ~6 $\times 10^5$ cells per well in whole media and incubated at 37℃ in a humidified, 5% CO_2 incubator for 18 h. *V. parahaemolyticus* was grown to 10^8 cells in 10 mL LB broth with 1% NaCl at 37℃ for 24 h, then centrifuged at 3000 rpm for 30 min and resuspended in 10 mL PBS. The Caco-2 cells were inoculated with ~8.0 log CFU of *V. parahaemolyticus* in whole media without (control) or with the respective SIC of EG or RH and incubated for 3 h. The infected

monolayers were rinsed three times with 1 mL PBS after 3 h of incubation, and the cells were lysed by treating with 1 mL 0.1% Triton X-100 (Sigma) for 15 min. The number of viable adherent *V. parahaemolyticus* was determined by serial 10-fold dilution and plating on TCBS agar plates. Duplicate samples were included and the experiment was repeated three times.

6. TDH/ Hemolysis assay

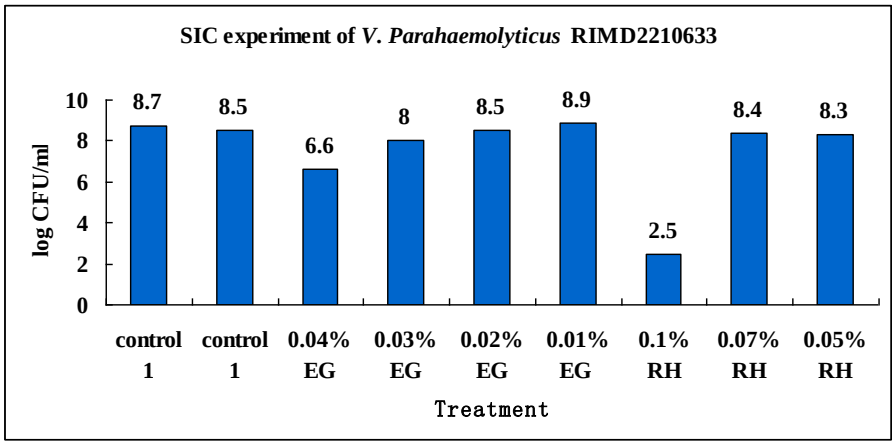
TDH activity in the supernatant of *V. parahaemolyticus* culture grown in the presence and absence of SIC of EG or RH was quantified by hemolysis assay, as described previously [101-103]. Overnight cultures of *V. parahaemolyticus* in 10 mL LB+1% NaCl with or without SIC of EG (0.03%) and RH (0.07%) were centrifuged at 3600 rpm for 20 min and the supernatant was collected. Defibrinated sheep blood (Quadfive, Ryegate, MT) was centrifuged at 1000 ×g for 10 min and the supernatant was discarded. The red blood cells (RBC) were washed three times and resuspended in 10 mL PBS at 3% concentration. One hundred µl of freshly prepared 3% SRBCs were added to each well. Positive hemolysis controls (100% hemolysis) obtained by addition of 100 µl of sterile distilled water and 100 µl of 3% SRBC and saline controls (0% hemolysis) obtained by addition of 100 µl of PBS to 100 µl of 3% SRBC were included. The plates were incubated for 30 min at 37℃. The absorbance was measured at 600 nm and percentage hemolysis was estimated according to the formula (% hemolysis = (1 − ODs / ODt) × 100, where ODs refers to the differences in optical density (600 nm) between the sample and positive control, and ODt refers to the differences in optical density (600 nm) between the saline control and positive control, respectively (Bhakdi et al., 1984).

7. Statistical analysis

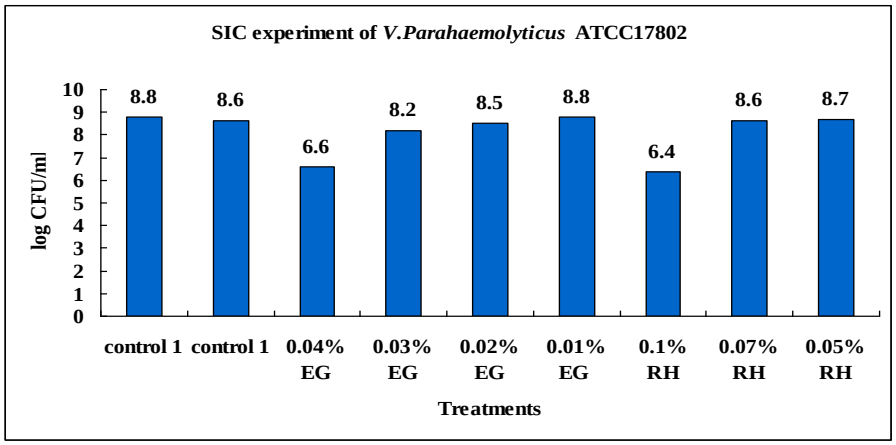
Data from the three independent replicate experiments were collected. The data ware analyzed by one-way ANOVA. Differences were considered significant at $P < 0.05$.

IV. RESULTS AND DISCUSSION

Based on the growth pattern of both strains of *V. parahaemolyticus* in the absence (control) or presence of plant compounds, the SIC of EG and RH was found to be 0.03% and 0.07%, respectively (Fig. 1A-B). EG at 0.03% and RH at 0.07% were highest concentration for each plant compounds that did not inhibit bacterial growth. Results showed that there was no significant difference in the populations of *V. parahaemolyticus* cultured in absence (control) or with 0.03% EG and 0.07% RH. After 24 hours incubation at 37℃, bacterial numbers reached to ~8 log CFU/ml in both treatment and control (Fig. 1A-B). Therefore 0.3% and 0.7% were selected as the SIC of EG and RH, respectively for subsequent experiments. Since SIC of antimicrobial does not affect the growth of bacteria, the effects observed in the phenotypic tests could be due to the effect of plant compounds on the expression of virulence factors associated with its pathogenesis.



(A)

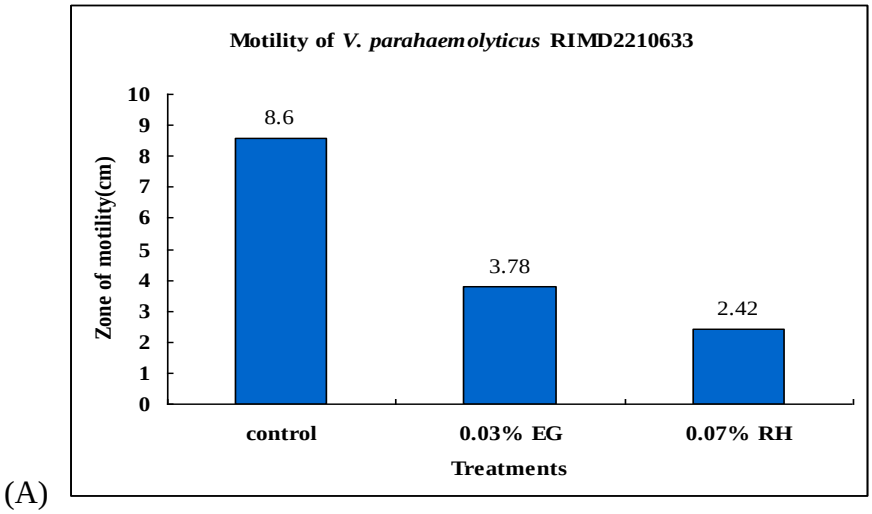


(B)

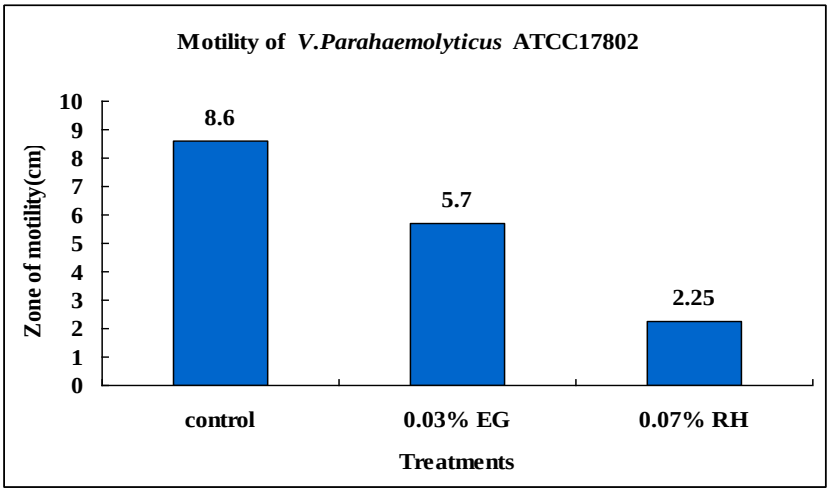
Figure 1. Effect of various concentrations of Eugenol (EG) and Rutin Hydrate (RH) on growth of (A) *V. Parahaemolyticus* RIMD2210633 and (B) ATCC17802. The

Y-axis represents bacteria in log CFU/ml and the X-axis represents the various treatments.

The effect of EG and RH on *V. parahaemolyticus* motility is shown in Fig.2A-B. Control had a zone of motility of approximately 8.6 cm after 18 h incubation at 37 ℃. Both EG and RH at SIC decreased *V. parahaemolyticus* motility as compared to control ($p<0.05$). The zone of motility of *V. parahaemolyticus* treated with EG was less than 4 cm, whereas when treated with RH the zone of motility reduced to less than 2.5 cm. Results showed that two plant-derived antimicrobials at their SIC reduced *V. parahaemolyticus* motility by 44% and 73%, respectively.



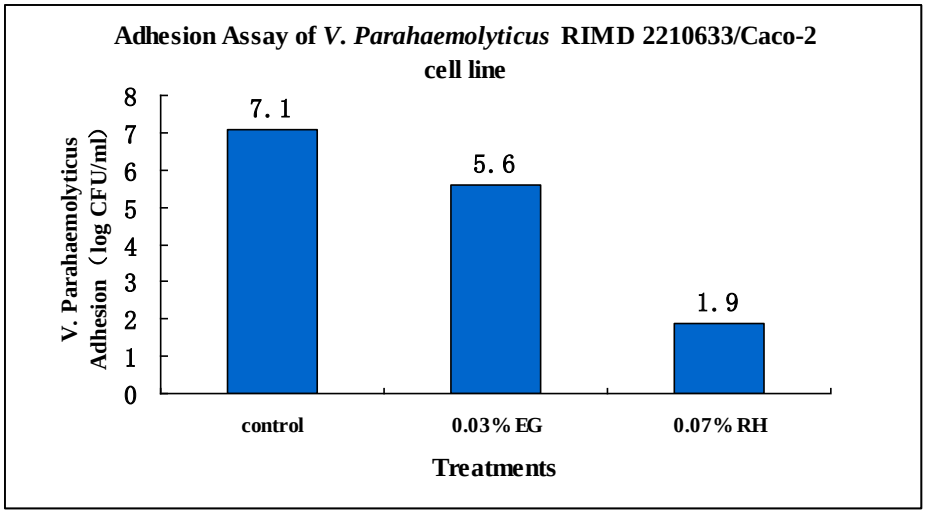
(A)



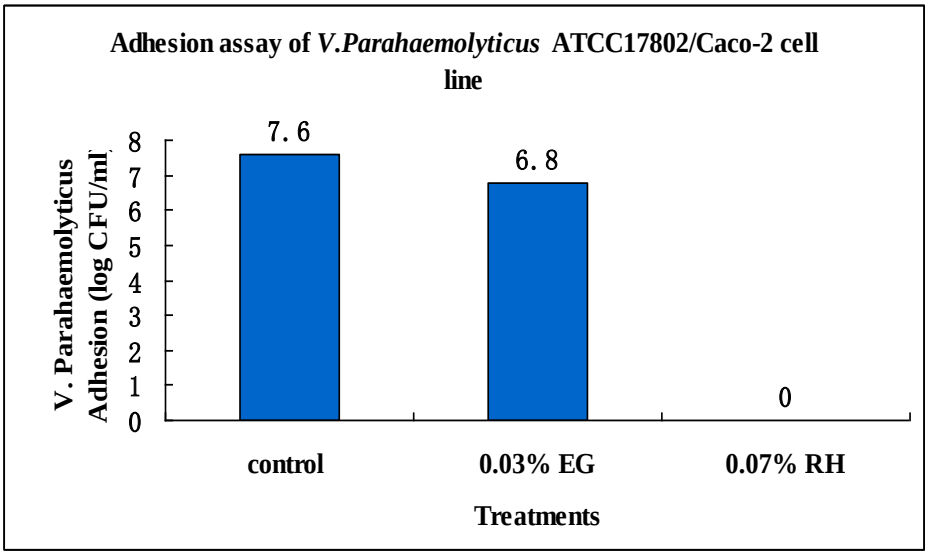
(B)

Figure 2. Effect of SIC of Eugenol (EG) and Rutin Hydrate (RH) on (A) *V. parahaemolyticus* RIMD2210633 and (B) ATCC17802 motility. The Y-axis represents zone of bacterial motility and the X-axis represents the various treatments.

Since attachment of enteric pathogen such as *V. parahaemolyticus* is critical for initiating infection in the host, we investigated the effect of EG and RH on *V. parahaemolyticus* attachment to Caco-2 cells. Cell culture studies showed that the two plant compounds reduced *V. parahaemolyticus* adhesion to Caco-2 cells by 15 to 85%, as compared to control ($p<0.05$) (Fig.3A-B). EG at 0.03% reduced adhesion of *V. parahaemolyticus* to Caco-2 cells by ~ 20% (Fig. 3A), whereas RH at its SIC of 0.07% reduced *V. parahaemolyticus* adhesion to Caco-2 cells by 80% (Fig.3A).



(A)



(B)

Figure 3. Effect of SIC of Eugenol (EG) and Rutin Hydrate (RH) on *V. parahaemolyticus* (A) RIMD2210633 (B) ATCC17802 adhesion to Caco-2 cells.

The effect of EG and RH on *V. parahaemolyticus* ATCC17802 hemolysin production is shown in Fig. 4. The hemolysis produced by sterile deionized water treatment was taken as 100% hemolysis and PBS treatment represented 0% hemolysis. In the case of control, approximately 55% hemolysis of sheep RBC was observed, whereas both EG and RH reduced hemolysis of sheep RBC by approximately 10 and 15% respectively, as compared to control ($P<0.05$).

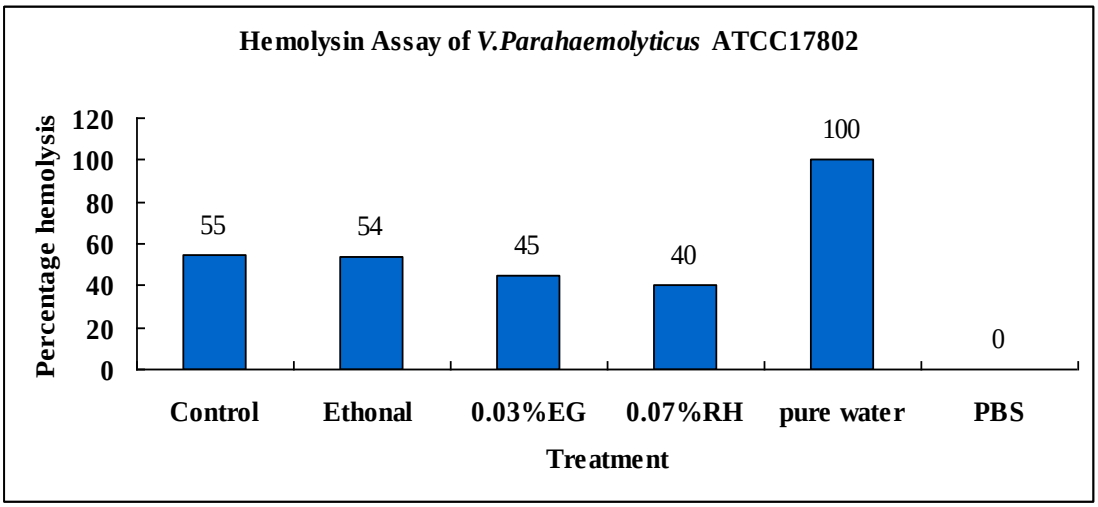


Figure 4. Effect of SIC of Eugenol (EG) and Rutin Hydrate (RH) on *V. parahaemolyticus* ATCC17802 hemolysis of sheep RBC.

V. CONCLUSION

In conclusion, our study demonstrated that the two plant-derived antimicrobials, EG and RH were effective in reducing the adhesion of both strains of *V. parahaemolyticus* to human intestinal cells *in vitro*. In addition, the plant compounds also significantly reduced bacterial motility and hemolysin production. Results suggest that EG and RH could potentially be used to reduce *V. parahaemolyticus* virulence factors critical for motility, hemolysin production and adhesion to intestinal epithelial cells, however, *in vivo* and gene expression studies are necessary to validate these results.

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反式肉桂醛抑制婴儿配方奶粉中的阪崎肠杆菌活性

Mary Anne Roshni Amalaradjou, Thomas A. Hoagland, Kumar Venkitanarayanan*

动物科学学院,康涅狄格大学,美国康涅狄格州 06269

摘要

阪崎肠杆菌是一种新兴的病原体， 它会造成脑膜炎，坏死性肠炎和脑炎，从而危及新生儿和儿童的生命。流行病学研究表明其主要来源为干燥的婴儿配方奶粉。反式肉桂醛是肉桂的树皮提取物中的主要成分。它被美国食品和药物管理局分类为一般确认为安全的（GRAS ）食品添加剂，并已被批准用于食物中添加使用（批准编号 21 CFR 182.60 ）。本研究的目的是确定反式肉桂醛对阪崎肠杆菌在重构的婴儿配方奶粉的抗菌作用。5 株混合的阪崎肠杆菌（6.0 log 菌落形成单位/毫升）接种到 10ml 含有 0 %，0.15 %，0.3%或 0.5%的反式肉桂醛的婴儿配方奶粉的样品中。将样品在 37℃，23℃， 8℃或 4℃分别培养 0 ，6，10 和 24 小时，阪崎肠杆菌的存活菌在每个采样时间进行了计数。此外，反式肉桂醛的潜在的细胞毒性，通过人胚胎肠细胞系（ INT- 407）进行了测定。含有反式 - 肉桂醛的治疗的阪崎肠杆菌的菌落数相对于对照组显著降低（ $P<0.05$）。添加反式肉桂醛（0.5%）分别在 37 ℃或 23 ℃培养 4 小时，和 8℃或 4℃培养 10 小时，降低病原体数目到检测不到的水平。通过对人类胚胎肠细胞在测试，该浓度的反式肉桂醛浓度无细胞毒害作用。结果表明，反式肉桂醛有可能被用来杀灭复原的婴儿配方奶粉中的阪崎肠杆菌，但是建议在使用前对其口感度进行研究。

关键词：阪崎肠杆菌 婴儿配方奶粉 反式肉桂醛 抑制活性

1、简介

干燥粉状的婴儿配方奶粉是送往世界各地喂养婴儿的主要配方奶粉，此产品以模仿人类母乳的营养价值而配制。婴儿配方奶粉不是完全无菌的产品，可作为有害病原体的潜在来源。此外，婴幼儿没有一个发达的免疫系统，更容易受到食物传播疾病的感染。因此，婴幼儿配方奶粉及食品的微生物安全性是至关重要的。为确保婴幼儿配方奶粉的微生物安全性，推荐对食品进行微生物测试，并与食品法典委员会设定的微生物标准进行比较。通常需要测试的微生物包括金黄色葡萄球菌，蜡样芽胞杆菌，阪崎肠杆菌和其它肠杆菌和沙门氏菌。在众多测试婴幼儿配方奶粉中存在的微生物中，阪崎肠杆菌被粮农组织和世界卫生组织定于 A 类重

点病源菌，婴幼儿配方奶粉同时也被认为是引起新生儿感染的一个潜在的媒介。

阪崎肠杆菌，是一种运动型，不形成孢子的革兰氏阴性兼性厌氧菌。这是一种报告造成 40-80 %新生儿病死率的新兴的病原体。它被认为是一种条件致病菌，通常污染婴幼儿配方奶粉，造成了罕见但是危及生命的新生儿脑膜炎，菌血症，坏死性肠炎和脑炎。除了病死率高，阪崎肠杆菌感染也可能会导致严重的神经系统后遗症，如脑积水，四肢瘫痪和幸存者迟钝神经发育。虽然阪崎肠杆菌的环境来源尚不清楚，流行病学研究表明干婴儿配方奶粉为传播的主要来源。这种细菌已被被众多研究者从婴幼儿配方奶粉中分离。此外，在美国还有许多因阪崎肠杆菌污染的婴儿配方奶粉被召回。 2002 年 11 月，据报道，超过 150 万罐沾染阪崎肠杆菌干婴儿配方奶粉在全国范围内召回。 2002 年 4 月 12 日，美国食品和药物管理局（ FDA）对医疗保健专业人士发布警示，关于与新生儿食用基于牛奶的婴幼儿配方奶粉中阪崎肠杆菌感染有关的危险。此外，国际微生物规范食品委员会已将阪崎肠杆菌定性为“严重危害的受限人群，危及生命或产生长期慢性后遗症”。

配制婴幼儿配方奶粉,作为一种营养丰富的培养基,能够在足够水供应,时间和温度有利条件时，支持细菌生长。因此，一旦再水化后，细菌生长和感染的唯一的限制条件是存储时间和温度。在这方面，阪崎肠杆菌具有几个特点，使得它成为一个成功的婴幼儿配方奶粉源性病原体。例如， Breeuwer 等。 （ 2003 ）发现阪崎肠杆菌有很高的耐渗透胁迫和干燥。阪崎肠杆菌可以生长在温度低至 5.5 ℃，这个温度多被用于家用冰箱。在 10℃和室温下，阪崎肠杆菌分别具有 14 小时和 45 分钟的倍增时间。分别在 25℃10 小时和 16 小时下存放后，摄入阪崎肠杆菌的相对危险性将增加 30 倍和 30000 倍。阪崎肠杆菌被发现在复原的婴儿配方奶粉中，具有很短的滞后时间和传代时间。配制配方奶粉的不当储存可允许其大幅增长。因此，一个有效的抗菌屏障涉入可能会潜在地降低婴儿食用被污染的重组婴幼儿配方奶粉后引发阪崎肠杆菌感染的可能性。

近年来,科学家们在利用由于对于合成的化合物的安全性顾虑天然抗菌物质的兴趣日益增加。在婴幼儿食品中选择使用抗菌药物时，这一点尤其显著。植物提取精油传统上被用来保存食品，以及提高食品风味。几种来源于植物的精油的抗菌性能已被证实，这些精油的多种活性成分也已确定。反式肉桂醛是肉桂的树皮提取物的主要组成部分。它被列为一般由 FDA 认可为安全（GRAS），并已被批准用于食品（21 CFR 182.60）。美国香料提取物制造商协会报道，在亚长期和长期研究中,反式肉桂醛在保守的估计摄取和和没有观察到的不良反应发生水平之间有大幅度的安全性。该报告还指出，反式肉桂醛无遗传毒性和致突变作用。虽然，已有报道表明，反式肉桂醛再合成实验室培养基中，对肉毒梭状芽孢杆菌，

金黄色葡萄球菌，大肠杆菌 O157 ： H7 和鼠伤寒沙门氏菌具有的抗菌活性;但是，它在特定的食品中的应用对于提高食品安全性还没有被研究的深度。本研究的目的是确定在复原的婴儿配方中，在不同贮藏温度下，反式肉桂醛灭活阪崎肠杆菌的功效。

2、材料与方法

2.1 细菌菌株和培养基

五株阪崎肠杆菌（ ATCC 51329 ， ES 2879 ， ES 4581 ， ES 4593 ， ES 4603 ）被用于研究。所有菌株，除 ATCC 51329 和 ES 2879 以外，均是从 Wybo Ingrid 博士处获得。ES 2879 是由杰弗里博士提供。所有菌株除 ATCC 51329 从婴幼儿配方或加工厂中分离得到。在研究中使用的所有细菌培养基购于 Difco。病原体的各菌株分别培养于加有 10ml 的无菌胰酶大豆肉汤（ TSB）的 30 毫升螺旋盖试管中，在 37 ℃下进行搅拌（ 150rpm）下培养 20 小时。孵育后，将培养物离心（8000 ×g，10 分钟）沉淀，用水洗涤两次，并重新悬浮于 10ml 无菌磷酸盐缓冲盐水（PBS ， pH 7.2）中。每个培养管中的细菌群体数的物通过连续稀释后，分别从每个浓度吸取 0.1 毫升菌液，涂于含有 0.6 %酵母提取物（TSAY）的胰蛋白酶大豆琼脂平板上，在 37 ℃下进行 24 小时。5 株阪崎肠杆菌同体积量混合，经适当稀释后，吸取 100μl 的悬浮液作为接种物。五铢混合细菌计数也通过电镀适当稀释 0.1 毫升部分上 TSAY 板，该平板在 37℃下孵育 24 小时后来证实。

2.2 反式肉桂醛

反式肉桂醛从 Sigma Chemical 公司购买。反式肉桂醛的抗菌效果于 0%（对照组）、0.15%、0.3% 和 0.5%下检测。

2.3 样品准备

婴幼儿配方奶粉的市售品牌从零售商店购买，并按照制造商的标签上的说明而重构。简言之，25.5 克奶粉在 180 毫升无菌蒸馏水中重新溶解，吸取 10ml 分别注入到 30ml 的螺丝帽的聚丙烯管中，并于 63℃巴氏消毒 30 分钟。反式肉桂醛加入到配方奶粉中，从而得到上述的检测浓度。

2.4 接种、孵育和决定其抗菌活性

100μl 的 5 株适当稀释的混合病原体分别加入到上述配方的样品中，获得约 7.0log CFU / ml 的接种水平。不添加反式肉桂醛接种牛奶样品作为对照。将接种的样品在 37℃，23℃，8℃或 4℃孵育 0，4，10 和 24 小时，细菌群体数的物通过连续稀释后(在 PBS 中 1:10)涂布于 TSAY 板上。坂崎肠杆菌通过将 TSAY 上的代表群落在紫红色胆汁葡萄糖琼脂平板（VRBGA）画线培养时观察到的特征

菌落形态来鉴定。当阪崎肠杆菌并非由直接涂平板检测时，样品于 100 毫升 TSB 37℃培养的 24 h 后，测试样品中的存活细胞数，随后于 VRBGA 上画线培养。每个控制组的重复样品以及对照组样品在每个指定的温度下培养，整个实验重复三次。另外在微生物分析中，每个处理和对照样品的 pH 值，也通过 Accumet pH 计测定。

2.5 细胞培养

人胚胎空肠和回肠衍生单层 INT-407 细胞在 37℃在 25 平方厘米的培养瓶中加有 5.0 毫摩尔的左旋谷氨酰胺和 10％热灭活的牛胎血清的伊格尔基础培养基中，于 5％CO2 和 95％空气的环境下进行培养。在细胞毒性测定前的细胞的存活率通过活体染料台盼蓝测定。

2.6 毒性测试

反式肉桂醛对 INT -407 细胞的细胞毒性潜能通过用 MTT 法检测培养至指数期细胞来确定。此方法是基于四唑鎓盐，四唑溴化物（MTT）通过活细胞的细胞的氧化还原酶还原成一种结晶蓝色的甲臜产物。所得到的甲臜晶体的形成与存活细胞的数量是成正比。在细胞活力测试中，INT- 407 细胞用胰蛋白酶消化至约 2-3×10^5 的细胞量接种到 96 孔组织培养板中，来提供 24 小时内的与样品的混合。之后将生长培养基换成新鲜全培养基，并分别加入 0.15 ％，0.3％或 0.5％的反式肉桂醛加入到实验组孔中。一组无反式肉桂醛的孔作为对照。每个实验组和对照组都有组内重复，该实验三次平行重复。24 小时孵育后，将细胞洗涤以除去测试化合物，并与 200μl 的新鲜培养基和 MTT（为 10mg/ml ）共同加入 10μl0.1M PBS，在 pH7.4，37 ℃，5％CO2 和 95％空气的环境下进行培养 4 小时。然后轻轻地从孔中吸出培养基，将 100μl 和洗涤剂加到所有孔中。然后将板保持在黑暗中 2 小时，吸光度用 Biorad 的酶标仪 550 在 570nm 处读取。除了细胞活力，细胞的形态，如单层细胞的损失，脱壁，萎缩，成粒和细胞质空泡等也通过显微镜检来观察改变。

2.7 数据分析

每个实验组和对照组的数据从重复的独立中汇集，并使用 GLM（一般线性模型）的统计分析软件的子程序进行分析。该模型对包括了实验组浓度，贮存温度和时间作为主要的影响。最少显著差异测试是用来确定细菌计数在不同实验组浓度和的不同存储温度下的显著差异（$P<0.05$）。

3、结果与讨论

重组后的婴幼儿配方奶粉的平均 pH 值为 6.78。加入 0.15%，0.3%和 0.5％的反式肉桂醛并没有导致奶粉的 pH 任何显著变化。阪崎肠杆菌于重组婴幼儿配方奶粉样品中的平均初始菌落数约为 7.0 log CFU/ml。37℃培养时，反式肉桂

醛对复原的婴儿配方奶粉中阪崎肠杆菌抑制效果如图 1A 所示。在培养 4 小时后，阪崎肠杆菌的菌群数减少到检测不到的水平（负富集）。此时加有 0.5%，0.3%和 0.15%的反式肉桂醛的实验组的菌落群分别为大约 5.0 log CFU/ml 和 6.0 log CFU/ml。在 24 小时结束时，含 0.3%反式肉桂醛样品检测不到阪崎肠杆菌，而含有 0.15 %反式肉桂醛有阪崎肠杆菌的约 3.5log CFU/ml。在不含反式肉桂醛的对照样品中病原体增长最终达到约 9.5log CFU/ml。反式肉桂醛在 23℃时对阪崎肠杆菌的抗菌作用与在 37℃时的观察相似（数据未显示）。

反式肉桂对阪崎肠杆菌醛在 8℃的效果如图 1B 所示。阪崎肠杆菌在含有 0.5%反式肉桂醛处理的样品于 8℃，10 小时培养后完全失活，而在 0.3%和 0.15%反式肉桂醛处理后只是分别降低了病原体计数约 2.5 log CFU/ml 和 1.5 log CFU/ml。在在 8℃ 孵育 24 h 后，0.3 和 0.15 %反式肉桂醛，分别降低阪崎肠杆菌计数约 3.0log CFU/ml 和 1.5log CFU/ml。反式肉桂醛在 4℃时对阪崎肠杆菌的抗菌作用与在 8℃时的观察相似（数据未显示）。在 8℃和 4℃下，0.5 %的反式肉桂醛在 10 小时内完全灭活的病原体，而 0.3 %和 0.15 %的反式肉桂醛分别降低阪崎肠杆菌的菌落约 3.0log CFU/ml 和 1.2log CFU/ml。在两个温度下，阪崎肠杆菌的对照组样品中的菌群数在整个 24 小时的贮存期并没有显著变化（P>0.05）。

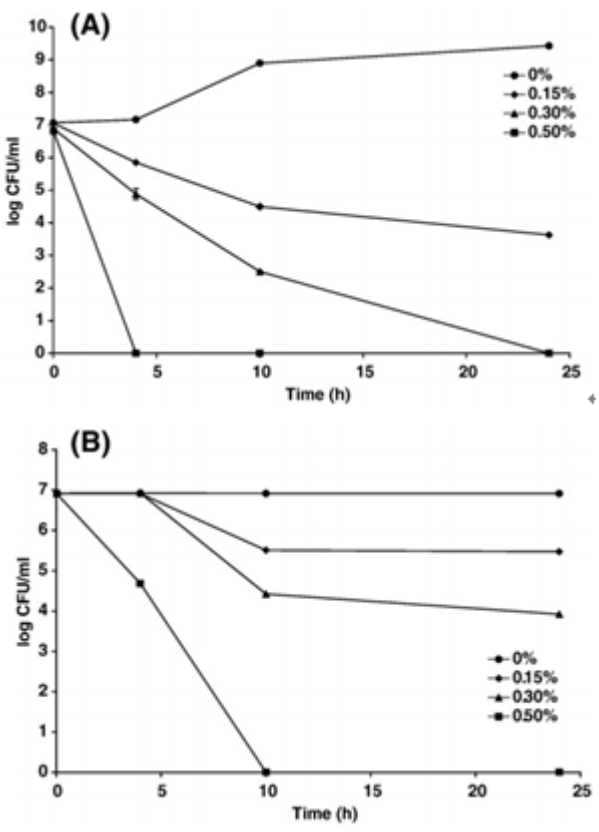

图 1. A.37 ℃时在婴儿复方奶粉中，反式肉桂醛对阪崎肠杆菌的影响。
B. 8 ℃时在婴儿复方奶粉中，反式肉桂醛对阪崎肠杆菌的影响。(●)对照组，(◆）0.15%反式肉桂醛，(▲)0.3%反式肉桂醛，(■）0.5% 反式肉桂醛.

通过 MTT 法进行测定，对照组相比，三种浓度的反式肉桂醛均未对 INT-407

细胞的存活率上有任何影响（数据未显示）。类似地，与对照组相比，反式肉桂醛处理后的 INT-407 细胞的显微镜检查结果也没有显示在形态学任何可检测的变化。这些结果表明，反式肉桂醛不产生对肠上皮细胞的任何有害影响。

反式肉桂醛对阪崎肠杆菌的抗菌显著效果（P＜0.05）随其浓度增加而增加。例如，在 37℃，0.5％的反式肉桂醛经 4 小时孵育，降低阪崎肠杆菌数目到检测不到的水平，而含有 0.3％反式肉桂醛的样品仅在孵育 24 小时导致的病原体完全失活（图 1A）。在 4℃，8℃和 23℃时，含 0.5%，0.3%和 0.15%的反式肉桂醛样品中阪崎肠杆菌的计数之间也有显著差异（P ＜0.05）。

观察到，相对于 23 和 37℃较低的温度（4 和 8℃）下，反式肉桂醛对阪崎肠杆菌减小了抑制作用。例如，0.5％反式肉桂醛在 37℃时，4 小时孵育便完全灭活阪崎肠杆菌（图 1A），而观察到在 8℃时，只在 10 小时才出现灭活的同一数量级（图 1B）。4℃和 8℃下的反式肉桂醛显著减少的抗菌作用可能是由于在低温下脂肪酸谱和细菌细胞的膜的流动性发生变化。质膜是反式肉桂醛的主要靶点，从而改变细胞膜的脂质组合物，包括改变不同的脂肪酸类的相对比例，并且增加暴露于寒冷时脂质的不饱和度和流动性。这些在细菌细胞膜上的改变可潜在干扰反式肉桂醛的作用效果，从而导致在低温下的抗菌作用下降。肉桂醛在 37℃和 21℃增加的抗菌效果可以归因于这些温度下，相比于 8℃或 4℃，阪崎肠杆菌有更高的代谢，生长率和死亡率。此前，Yuste 及 fung 还观察到, 乳酸链球菌素和肉桂的组合在 20℃相比于 5℃对苹果汁中的大肠杆菌 O157：H7 和沙门氏菌有更高的灭火率。

疾病控制和预防中心（CDC）建议，重组婴儿配方奶粉应冷藏，并在 24 小时冷冻后丢弃，同时准备好的配方奶粉不宜在室温下存放超过 4 小时。本研究表明，反式肉桂醛在配方奶粉室温下储存时，对 7.0 log CFU/ml 阪崎肠杆菌快速抑菌作用，4 小时内用有 0.5%的计数值减少。在 4℃冷藏储存 10 小时，0.5％的反式肉桂醛有效降低阪崎肠杆菌超过 6.0 log CFU/ml。这些发现表明，反式肉桂醛在室温和冷藏温度于阪崎肠杆菌均有显著的抗菌活性,在 CDC 建议的存储期限内杀死大量的病原体。

4、结论

本研究结果表明，反式肉桂醛可以抑制在婴幼儿配方奶粉中新生儿病原体的活性。所有测试浓度下的反式肉桂醛均对人类胚胎肠道细胞无毒性作用。因此，将反式肉桂醛在婴幼儿配方奶粉掺入，是一种保护从婴儿通过阪崎肠杆菌污染的配方奶粉从而获得感染的可行方法。然而，在建议其作为抗菌成分在使用补充入婴幼儿配方奶粉前，需要对反式肉桂醛进行详细的感官研究。进一步的研究还需要保证反式肉桂醛对婴幼儿的内源性菌群的没有影响。

声明

本人声明所呈交的学位论文是本人在导师指导下进行的研究工作及取得的研究成果。据我所知，除了文中特别加以标注和致谢的地方外，论文中不包含其他人已经发表或撰写过的研究成果，也不包含为获得四川大学或其他教育机构的学位或证书而使用过的材料。与我一同工作的同志对本研究所做的任何贡献均已在论文中作了明确的说明并表示谢意。

本学位论文成果是本人在美国康涅狄格大学交流期间，在院内院外导师共同指导下取得的，论文成果归四川大学所有，特此声明。

导　师：

本科生：

日　期：2014 年 5 月

致谢

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附录—翻译原文

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Inactivation of *Enterobacter sakazakii* in reconstituted infant formula by trans-cinnamaldehyde

Mary Anne Roshni Amalaradjou, Thomas A. Hoagland, Kumar Venkitanarayanan *

Department of Animal Science, Unit-4040, University of Connecticut, Storrs, Connecticut 06269, USA

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ABSTRACT

Enterobacter sakazakii is an emerging pathogen which causes a life-threatening form of meningitis, necrotizing colitis and meningoencephalitis in neonates and children. Epidemiological studies implicate dried infant formula as the principal source of the pathogen. Trans-cinnamaldehyde is a major component of bark extract of cinnamon. It is classified as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration, and is approved for use in food (21 CFR 182.60). The objective of this study was to determine the antibacterial effect of trans-cinnamaldehyde on *E. sakazakii* in reconstituted infant formula. A 5-strain mixture of *E. sakazakii* was inoculated into 10 ml samples of reconstituted infant formula (at 6.0 log CFU/ml) containing 0%, 0.15%, 0.3% or 0.5% trans-cinnamaldehyde. The samples were incubated at 37, 23, 8 or 4 °C for 0, 6, 10 and 24 h, and the surviving populations of *E. sakazakii* at each sampling time were enumerated. In addition, potential cytotoxicity of trans-cinnamaldehyde, if any, was determined on human embryonic intestinal cells (INT-407). The treatments containing trans-cinnamaldehyde significantly reduced ($P<0.05$) the population of *E. sakazakii*, compared to the controls. Trans-cinnamaldehyde (0.5%) reduced the pathogen to undetectable levels by 4 h of incubation at 37 or 23 °C and 10 h of incubation at 8 or 4 °C, respectively. Trans-cinnamaldehyde produced no cytotoxic effects on human embryonic intestinal cells at the tested concentrations. Results indicate that trans-cinnamaldehyde could potentially be used to kill *E. sakazakii* in reconstituted infant formula, however sensory studies are warranted before recommending its use.

1. Introduction

Powdered infant formula constitutes the majority of infant formula fed to infants world wide (Drudy et al., 2006). This product is formulated to mimic the nutritional profile of human breast milk (Breeuwer et al., 2003). Powdered infant formula is not a sterile product and can act as a potential source of harmful pathogens. In addition, infants and young children do not have a well developed immune system and hence are more vulnerable to food-borne infections. Therefore the microbiological safety of the infant and follow-up formula is critical. To assure the microbiological safety of infant formula, several microbiological tests are recommended and compared with the microbiological criteria set by the Codex Alimentarius Commission (CAC, 1979). The specific microbes commonly tested include *Staphylococcus aureus*, *Bacillus cereus*, *Enterobacter sakazakii* and other Enterobacteriaceae and *Salmonella* (Forsythe, 2005). Among the specific microbes tested for presence in infant formula, *E. sakazakii* is placed under category A by FAO–WHO (FAO/WHO, 2004) and is considered to be a potential agent for causing neonatal infections.

E. sakazakii, is a motile, non-sporeforming gram-negative facultative anaerobe. It is an emerging neonatal pathogen with a reported case fatality rate of 40–80% (Bowen and Braden, 2006). It is considered to be an opportunistic pathogen that contaminates powdered infant formula, causing a rare, but life-threatening form of neonatal meningitis, bacteremia, necrotizing colitis and meningoencephalitis (Kleiman et al., 1981; Nazarowec-White and Farber, 1997b; Sanders and Sanders, 1997; van Acker et al., 2001). In addition to the high fatality rate of *E. sakazakii* infections, it may result in severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development in survivors (Forsythe, 2005). Although the environmental source of *E. sakazakii* is not clearly understood, epidemiological studies implicate dried infant formula as the primary source of transmission (Simmons et al., 1989; van Acker et al., 2001; Weir, 2002). The bacterium has been isolated from powdered infant formula by numerous investigators (Postupa and Aldova, 1984; Muytjens et al., 1988; Biering et al., 1989; Simmons et al., 1989; Muytjens and Kollee, 1990). Moreover, there were many recalls of *E. sakazakii*-contaminated infant formula in the United States. In November 2002, a nationwide recall of more than 1.5 million cans of dry infant formula contaminated with *E. sakazakii* was reported (FSNET, 2002). On April 12, 2002, the United States Food and Drug Administration (FDA) issued an alert to U.S. health care professionals

* Corresponding author. Tel.: +1 860 486 0947; fax: +1 860 486 4375.
E-mail address: kumar.venkitanarayanan@uconn.edu (K. Venkitanarayanan).

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regarding the risk associated with *E. sakazakii* infections among neonates fed milk-based, powdered infant formula (FDA, 2002). In addition, the International Commission on Microbiological Specification for Foods (ICMSF, 2002) has ranked *E. sakazakii* as 'Severe hazard for restricted populations, life-threatening or substantial chronic sequelae of long duration'.

Being a nutrient-rich medium, reconstituted powdered infant formula can support bacterial growth when favorable conditions of water availability, time and temperature are provided. Therefore once rehydrated the only limiting conditions for bacterial growth and infection are storage time and temperature. In this regard, *E. sakazakii* possesses several characteristics that enable it to be a successful infant formula-borne pathogen. For example, Breeuwer et al. (2003) revealed that *E. sakazakii* has a high tolerance to osmotic stress and desiccation. *E. sakazakii* can grow at temperatures as low as 5.5 °C (Nazarowec-White and Farber, 1997c), which has been reported to be the temperature of many home refrigerators (Harris, 1989). *E. sakazakii* has a doubling time of 14 h and 45 min at 10 °C and room temperature, respectively (Iversen et al., 2004). The relative risk of ingesting *E. sakazakii* will increase by 30-fold and 30,000-fold after storage for 10 h at 25 °C and 16 h at 25 °C, respectively. *E. sakazakii* was found to possess a short lag time and generation time in reconstituted infant formula (Nazarowec-White and Farber, 1997a), raising concerns that improper storage of reconstituted formula may permit its substantial growth. Therefore, incorporation of an effective antimicrobial barrier may potentially reduce the likelihood of outbreaks of *E. sakazakii* infection in infants through ingestion of contaminated reconstituted infant formula.

In recent years, there has been an increasing interest in the use of natural antimicrobial substances due to concerns regarding the safety of synthetic compounds (Abee et al., 1995). This is especially significant when selecting antimicrobials for use in infant foods. Plant-derived essential oils have been traditionally used to preserve foods as well as enhance food flavor. The antimicrobial properties of several plant-derived essential oils have been demonstrated (Bilgrami et al., 1992; Burt, 2004; Holley and Patel, 2005), and a variety of active components of these oils have been identified. Trans-cinnamaldehyde is a major component of bark extract of cinnamon. It is classified as generally recognized as safe (GRAS) by the FDA, and is approved for use in foods (21 CFR 182.60). The U. S. Flavoring Extract Manufacturers' Association reported that trans-cinnamaldehyde has a wide margin of safety between conservative estimates of intake and no observed adverse effective levels, from sub chronic and chronic studies (Adams et al., 2004). The report also indicated no genotoxic and mutagenic effects due to trans-cinnamaldehyde. Although the antibacterial activity of trans-cinnamaldehyde has been reported against *Clostridium botulinum* (Bowles and Miller, 1993), *S. aureus* (Bowles et al., 1995), *E. coli* O157:H7 and *Salmonella typhimurium* (Helander et al., 1998) in synthetic laboratory media; its application in specific foods for improving food safety has not been investigated in depth. The objective of this study was to determine the efficacy of trans-cinnamaldehyde for inactivating *E. sakazakii* in reconstituted infant formula at different storage temperatures.

2. Materials and methods

2.1. Bacterial strains and media

Five strains of *E. sakazakii* (ATCC 51329, ES 2879, ES 4581, ES 4593, ES 4603) were used for the study. All the strains, excepting ATCC 51329 and ES 2879 were obtained from Dr. Wybo Ingrid, Department of Microbiology, Academisch Ziekenhuis Vrije Universiteit Brussel, Brussels, Belgium. The isolate ES 2879 was provided by Dr. Jeffrey. M. Farber, Bureau of Microbial Hazards, Sir Frederick Banting Research Center, Ottawa, Canada. All the strains with the exception of ATCC 51329 were isolated from infant formula or processing plants. All bacteriological media used in the study were procured from Difco (Sparks, Md.). Each strain of the pathogen was cultured separately in 10 ml of sterile tryptic soy broth (TSB) in 30-ml screw-cap tubes at 37 °C for 20 h with agitation (150 rpm). Following incubation, the cultures were sedimented by centrifugation (8000 ×*g* for 10 min), washed twice, and resuspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.2). The bacterial population in each culture was determined by plating 0.1-ml portions of appropriately diluted culture on duplicate tryptic soy agar plates containing 0.6% yeast extract (TSAY) with incubation at 37 °C for 24 h. Equal portions from each of the five strains were combined, and 100 μl of the appropriately diluted suspension was used as the inoculum. The bacterial count of the five-strain mixture of the pathogen was also confirmed by plating 0.1-ml portions of appropriate dilutions on TSAY plates, and incubating the plates at 37 °C for 24 h.

2.2. Trans-cinnamaldehyde

Trans-cinnamaldehyde was purchased from Sigma chemical Co. (St. Louis, Mo.). The antimicrobial effect of trans-cinnamaldehyde was tested at 0% (control), 0.15%, 0.3% and 0.5%.

2.3. Sample preparation

A commercially available brand of powdered infant formula was purchased from a retail store and reconstituted as per the manufacturer's instructions on the label. Briefly, 25.5 g of the formula was reconstituted in 180 ml of sterile distilled water, 10 ml volumes were dispensed into 30-ml screw capped polypropylene tubes, and pasteurized at 63 °C for 30 min. Trans-cinnamaldehyde was added to the formula to obtain the aforementioned final concentrations.

2.4. Inoculation, incubation and determination of antibacterial activity

A volume of 100 μl of the appropriately diluted five-strain mixture of the pathogen was separately added to the formula samples to obtain an inoculation level of approximately 7.0 log CFU/ml. Inoculated milk samples without any added trans-cinnamaldehyde served as controls. The inoculated milk samples were incubated at 37, 23, 8 or 4 °C for 0, 4, 10 and 24 h, and the surviving populations of the pathogen were enumerated by plating directly or after serial dilutions (1:10 in PBS) on duplicate TSAY plates. Representative colonies on TSAY were confirmed as *E. sakazakii* based on the characteristic colonial morphology observed when streaked on violet red bile glucose agar plates (VRBGA). When *E. sakazakii* was not detected by direct plating, samples were tested for surviving cells by enrichment for 24 h at 37 °C in 100 ml of TSB, followed by streaking on VRBGA. Duplicate samples of each treatment and control were included at each of the specified temperatures, and the entire study was replicated three times. In addition to microbiological analyses, the pH of each treatment and control sample was also determined using an Accumet pH meter (Fisher Scientific, Pittsburgh, Pa.).

2.5. Cell culture

Monolayers of INT-407 cells (ATCC, Manasas, Va), derived from human embryonic jejunum and ileum, were cultured at 37 °C in 25-cm^2 flasks and grown in basal medium eagle (BME, Gibco BRL) supplemented with 5.0 mM L-glutamine and 10% heat inactivated fetal calf serum (Sigma, St. Louis, MO) in an atmosphere of 5% CO_2 and 95% air. The viability of the cells prior to the cytotoxicity assay was confirmed by exclusion of the vital dye trypan blue (Pazos et al., 2002).

2.6. Cytotoxicity assay

The cytotoxic potential of trans-cinnamaldehyde on INT-407 cells was determined following incubation of exponentially growing cells using the MTT assay (Mosmann, 1983). This method is based on the reduction of the tetrazolium salt, methylthiazolyldiphenyl-tetrazolium bromide (MTT) into a crystalline blue formazan product by the cellular

oxidoreductases of viable cells (Mosmann, 1983). The resultant formazan crystal formation is proportional to the number of viable cells. For cell viability tests, INT-407 cells were trypsinized and approximately 2–3×10^5 cells were inoculated into wells of a 96-well tissue culture plate to provide confluence in 24 h. Thereafter the growth medium was replaced with fresh whole medium and 0.15%, 0.3% or 0.5% trans-cinnamaldehyde was added to the treatment wells. A set of wells without trans-cinnamaldehyde was used as controls. A total of three replicates were used for each treatment and control and the experiment was duplicated. Following 24-h incubation, cells were washed to remove test compounds and were then incubated with 200 µl of fresh culture media and 10 µl of MTT (10 mg/ml) in 0.1 M PBS, pH 7.4 at 37 °C in an atmosphere with 5% CO_2 for 4 h. The medium was then gently aspirated from wells and 100 µl of detergent reagent was added to all wells. The plates were then kept in the dark for 2 h and the absorbance was read at 570 nm using the Bio-RAD microplate reader 550 (Bio-Rad laboratories, Hercules, Ca) In addition to cell viability, the morphology of the cells was also inspected for microscopically detectable alterations such as loss of monolayer, rounding, shrinking of cells, granulations, and vacuolation in the cytoplasm.

2.7. Statistical analysis

For each treatment and control, the data from independent replicate trials were pooled, and analyzed using the GLM (general linear model) sub-routine of the statistical analysis software (SAS, 1987). The model included the treatment concentrations, storage temperature and time as the major effects. Least significant difference test was used to determine significant differences ($P<0.05$) due to treatment concentrations and storage temperatures on bacterial counts.

3. Results and discussion

The mean pH of the reconstituted infant formula was 6.78. The addition of 0.15, 0.3 and 0.5% trans-cinnamaldehyde did not result in any significant change in the pH of formula. The average initial population of *E. sakazakii* in the reconstituted infant formula samples was approximately 7.0 log CFU/ml. The effect of trans-cinnamaldehyde on *E. sakazakii* in reconstituted infant formula at 37 °C is shown in Fig. 1A. At 4 h of incubation, the population of *E. sakazakii* was reduced to undetectable levels (enrichment negative) and to approximately 5.0 log CFU/ml and 6.0 log CFU/ml by 0.5%, 0.3% and 0.15% trans-cinnamaldehyde, respectively. At the end of the 24 h, samples containing 0.3% trans-cinnamaldehyde showed no detectable *E. sakazakii* whereas those containing 0.15% trans-cinnamaldehyde had approximately 3.5 log CFU/ml of *E. sakazakii*. In the control samples devoid of trans-cinnamaldehyde, the pathogen grew, reaching a final population of approximately 9.5 log CFU/ml. The antibacterial effect of trans-cinnamaldehyde on *E. sakazakii* at 23 °C was similar to that observed at 37 °C (data not shown).

The effect of trans-cinnamaldehyde on *E. sakazakii* at 8 °C is shown in Fig. 1B. At 10 h of storage at 8 °C, *E. sakazakii* in the treatment samples containing 0.5% trans-cinnamaldehyde was completely inactivated, whereas 0.3 and 0.15% trans-cinnamaldehyde reduced the pathogen count approximately by 2.5 log CFU/ml and 1.5 log CFU/ml, respectively. At 24 h of incubation at 8 °C, 0.3 and 0.15% trans-cinnamaldehyde decreased *E. sakazakii* counts by approximately 3.0 log CFU/ml and 1.5 log CFU/ml, respectively. The antibacterial effect of trans-cinnamaldehyde on *E. sakazakii* at 4 °C was similar to that observed at 8 °C (data not shown). At both 8 and 4 °C, 0.5% trans-cinnamaldehyde completely inactivated the pathogen by 10 h, whereas 0.3% and 0.15% trans-cinnamaldehyde reduced *E. sakazakii* populations by approximately 3.0 log CFU/ml and 1.2 log CFU/ml, respectively. At both temperatures, the population of *E. sakazakii* in the control samples did not vary significantly ($P>0.05$) throughout the 24-h storage period.

The three concentrations of trans-cinnamaldehyde tested did not result in any reduction in the viability of INT-407 cells when compared to the controls, as tested by the MTT assay (data not shown). Similarly, microscopic examination of trans-cinnamaldehyde-treated INT-407 cells did not reveal any detectable change in the morphology when compared to the control. These results suggest that trans-cinnamaldehyde did not produce any deleterious effect on intestinal epithelial cells.

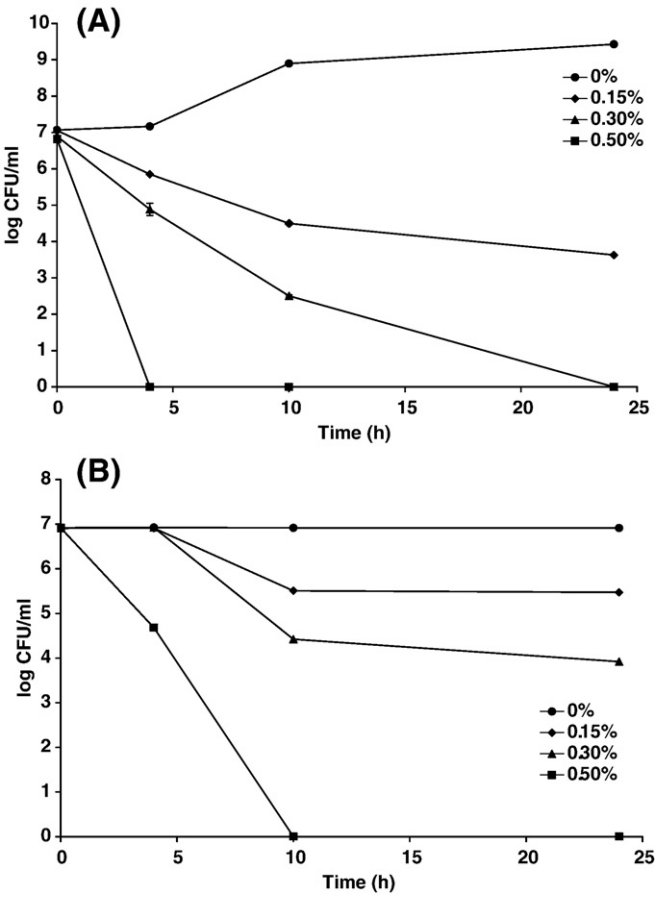

Fig. 1. A. Effect of trans-cinnamaldehyde on *E. sakazakii* in reconstituted infant formula at 37 °C. B. Effect of trans-cinnamaldehyde on *E. sakazakii* in reconstituted infant formula at 8 °C. (●) control, (♦) 0.15% trans-cinnamaldehyde, (▲) 0.3% trans-cinnamaldehyde, (■) 0.5% trans-cinnamaldehyde.

The antibacterial effect of trans-cinnamaldehyde on *E. sakazakii* significantly ($P<0.05$) increased with increase in its concentration. For example, at 37 °C, 0.5% trans-cinnamaldehyde reduced *E. sakazakii* counts to undetectable levels within 4 h of incubation, whereas samples containing 0.3% trans-cinnamaldehyde resulted in complete inactivation of the pathogen only at 24 h of incubation (Fig. 1A). Significant differences ($P<0.05$) in the counts of *E. sakazakii* between samples containing 0.5, 0.3 and 0.15% trans-cinnamaldehyde were also observed at 4 °C, 8 °C and 23 °C.

A reduced inhibitory effect of trans-cinnamaldehyde on *E. sakazakii* was observed at lower temperatures (4 and 8 °C) compared to 23 and 37 °C. For example, 0.5% trans-cinnamaldehyde completely inactivated *E. sakazakii* at 4 h of incubation at 37 °C (Fig. 1A), whereas the same magnitude of inactivation was observed at 8 °C only at 10 h (Fig. 1B). The significantly reduced antibacterial effect of trans-cinnamaldehyde at 4 and 8 °C could be due to the changes in the fatty acid profile and fluidity of bacterial cell membrane at cold temperatures. The plasma membrane is the primary target of trans-cinnamaldehyde (Gill and Holley, 2006) and changes in cell membrane lipid composition, including alterations in the relative proportions of different fatty acid classes, and increased lipid unsaturation and fluidity up on exposure to cold are reported (McElhaney, 1976; Russel, 1984). These changes in bacterial cell membrane may potentially interfere with the action of trans-cinnamaldehyde, thus resulting in a reduced antibacterial effect at lower

temperatures. The increased antibacterial effect of cinnamaldehyde at 37 and 21 °C could be attributed to the higher metabolic, growth and death rates of *E. sakazakii* at these temperatures compared to those at 8 or 4 °C. Previously, Yuste and Fung (2004) also observed a higher rate of inactivation of *E. coli* O157:H7 and *S. typhimurium* in apple juice by a combination of nisin and cinnamon at 20 °C compared to 5 °C.

The centers for disease control and prevention (CDC) recommends that reconstituted infant formula should be refrigerated and discarded after 24 h of refrigeration, and prepared formula should not be stored at room temperature for more than 4 h (Baker, 2002). It is evident from this study that trans-cinnamaldehyde exerted a rapid antimicrobial effect on *E. sakazakii* with 0.5% reducing its count by 7.0 log CFU/ml at 4 h of storage at room temperature. At 4 °C, 0.5% trans-cinnamaldehyde was effective in reducing *E. sakazakii* by more than 6.0 log CFU/ml at 10 h of refrigerated storage. These findings demonstrated that trans-cinnamaldehyde exerted significant antimicrobial activity on *E. sakazakii* both at room and refrigeration temperatures, killing substantial populations of the pathogen within the storage periods recommended by the CDC.

4. Concluding remarks

Results of this study indicate that trans-cinnamaldehyde can exert inhibitory activity against neonatal pathogens in infant formula preparations. All the tested concentrations of trans-cinnamaldehyde produced no cytotoxic effects on human embryonic intestinal cells. Therefore incorporation of trans-cinnamaldehyde in infant formula preparations would be a feasible way of protecting infants from *E. sakazakii* infections transmitted through contaminated formula. However, detailed sensory studies of trans-cinnamaldehyde-supplemented infant formula need to be conducted before recommending its use as an antimicrobial ingredient. Further research is also warranted on the effect of trans-cinnamaldehyde on the endogenous microflora in infants.

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