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Tea and soybean extracts in combination with milk fermentation inhibit growth and enterocyte adherence of selected foodborne pathogens

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ABSTRACT

This study examined the antibacterial and anti-adhesive properties of pure plant extracts (PPEs) of green tea (GT), black tea (BT) and soybean individually or in combination with milk. Fermented phenolic enriched-milk (fPEM) was prepared by combining PPEs with milk and fermented with lactic acid bacteria. Antimicrobial activity of extracts was evaluated by broth-dilution and agar diffusion assay. Anti-adhesive property of extracts was evaluated in Caco-2 cell model. Results from antibacterial tests showed that PPEs exhibited a dose-dependent growth inhibitory effect. Tea extracts were more effective in inhibiting Gram-positive bacteria while soybean extract exhibited similar effects against all pathogens tested. For fPEM, although total phenolic contents decreased compared with those in PPEs, growth inhibitory effect of fPEM containing tea extracts was greatly enhanced. All extracts showed significant inhibition against pathogen adhesion to Caco-2 cells. In particular, adhesion inhibition against *Staphylococcus aureus* and *Listeria monocytogenes* was >89% when fPEM extracts were applied.

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1. Introduction

Infections due to food-borne contamination by bacteria bring serious challenges to both food industries and consumers. Some of the bacterial infections can lead to serious and even fatal diseases: the enterohemorrhagic *Escherichia coli* O157:H7, which has been linked to large outbreaks of gastrointestinal illness due to the consumption of contaminated food as well as contact transmission (Vogt & Dippold, 2002); *Cronobacter sakazakii* is an opportunistic pathogen that exists in a wide range of food products and is now recognized as a growing threat to neonates and infants, and even immune compromised adults (Wakabayashi, Yamauchi, & Takase, 2008); *Staphylococcus aureus* can present as commensal flora in human beings, but may become pathogenic, resulting in skin infections, abscesses and even life-threatening diseases (Bhatia & Zahoor, 2007); *Listeria monocytogenes* is able to grow at low temperatures with wide distribution in the environment, and is the causative agent of listeriosis, which is one of the most fatal diseases (Carpentier & Cerf, 2011). Antibiotics solve part of the problem, but their side-effects cannot be underestimated and the gradually-developed drug resistance of some pathogens makes it an ever more pressing global problem. Moreover, consumers today desire food products that are natural, safe and health-promoting. A call has therefore been made to find or develop antimicrobials of natural origins.

Plant phenolic compounds, especially dietary phenolics, are attracting more attention due to their multiple health benefits and abundant sources. For example, tea catechins and soy isoflavones, classified as flavonoids, possess potent antioxidant, antimutagenic, anti-inflammatory and anti-hypertensive capacity. Among these bioactivities, antimicrobial and anti-adhesive properties are of particular interest for prevention of infectious disease.

Phenolics derived from tea (*Camellia sinensis* var. *sinensis* for Chinese teas) and soybean [*Glycine max* (L.) Merrill] are reported to inhibit the growth of foodborne pathogens including *S. aureus*, *E. coli*, *Bacillus cereus*, and *Helicobacter pylori*, but not the health-promoting bacteria such as lactic acid bacteria (LAB) and bifidobacteria (Ankolekar et al., 2011; Bansal et al., 2013). The modes of action of phenolic compounds, as reviewed previously (Cushnie & Lamb, 2011), are manifold. In particular, flavan-3-ols from tea are known to inhibit cell wall formation or cell division, damage cytoplasmic membrane, inhibit energy metabolism and interrupt biosynthetic protein expression, while isoflavone aglycones from soyfoods mainly inhibit nucleic acid synthesis and interrupt protein synthesis. In addition, their effectiveness in reducing the risk of intestinal infection is also reflected in the adhesion-inhibitory properties against some pathogens to epithelial cells, as adhesion to host is a primary

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requirement for the initiation of systematic infections. Since anti-adhesion therapy aims to only interrupt bacterial adhesion to host cells rather than kill them, antibiotic-like resistance towards the therapeutic agents is less likely to evolve (Huttunen, Toivanen, Arkko, Ruponen, & Tikkanen-Kaukanen, 2011).

Noticeably, combination of antibacterial agents of different origins can exhibit synergistic effects as they act through multiple mechanisms (Nazer, Kobilinsky, Tholozan, & Dubois-Brissonnet, 2005; Sivarooban, Hettiarachchy, & Johnson, 2008). LAB, as the major group of bacteria responsible for milk fermentation, have been reported to produce antibiotic-like substances (Dave & Shah, 1997). In addition, the breakdown of milk protein due to bacterial proteinase activity during fermentation generates antagonistic peptides that deter bacterial growth or inhibit bacterial adhesion to human enterocytes (Fitzgerald & Murray, 2006). In view of this, it would be interesting to develop a functional food with antibacterial and anti-adhesion activity by combining LAB-milk fermentation and dietary plant extracts. In this study, LAB-fermented milk enriched with the extract of three commonly consumed foodstuffs, i.e. green tea (GT), black tea (BT) and soybean (SB), was formulated as a novel functional beverage. The ability of the products to inhibit bacterial growth and to block bacterial adhesion to Caco-2 cell monolayers was examined after further processing. We also evaluated the antibacterial potential of pure plant extracts (PPEs) individually for comparison. *S. aureus* and *L. monocytogenes* were selected as the representative Gram-positive pathogenic bacteria and *E. coli* and *C. sakazakii* as the representative Gram-negative pathogenic bacteria. A probiotic bacterium, *Lactobacillus plantarum* WCFS1 was selected as a reference microorganism.

2. Materials and methods

2.1. Microorganisms and culture conditions

2.1.1. LAB strains

Streptococcus thermophilus ASCC 1275, *L. plantarum* WCFS1, *L. plantarum* ASCC 276 and *L. plantarum* ASCC 292 were stored at −80 °C. For activation, 10 ml aliquots of sterile MRS broth were inoculated with 2% (v/v) of each organism and incubated at 37 °C for 18 h. After the second transfer in MRS broth, all of the activated organisms except *L. plantarum* WCFS1 were transferred into reconstituted skim milk (RSM) and incubated at 37 °C for 18 h. RSM was prepared by dissolving 12% (w/v) skim milk powder in double deionized water and pasteurized at 120 °C for 15 min. The pH value of autoclaved RSM was 6.5. After the second transfer into RSM, bacterial stock was used for fermentation. Probiotic *L. plantarum* WCFS1 (in MRS stock) was used as a reference strain in antibacterial assays and anti-adhesion assay.

2.1.2. Pathogenic strains

Four food-borne pathogens including *E. coli* O157:H7 PELI 0480, *C. sakazakii* ATCC 29544, *S. aureus* CMCC 26003 and *L. monocytogenes* CMCC 54001 were used. Bacterial cultures were cultivated in LB broth for 18–20 h at 37°C prior to antimicrobial assays.

2.2. Preparation of pure plant extracts (PPEs) from tea leaves and soybean

2.2.1. Tea leaves

Green tea (Zhuyeqing, Sichuan Province, PRC) and black tea (Dianhong, Yunnan Province, PRC) leaves were purchased from tea retailers. Tea extract (TE) was prepared as per the method of Zhao and Shah (2014a) with minor modifications. Briefly, tea powder (2%, w/v, strength of "a cup of tea" according to Yam, Shah, & Hamilton-Miller, 1997) was infused in boiling deionized water (DW) for 10 min. Green tea extract (GTE) and black tea extract (BTE) were produced by first suction-filtering through triple-layered Whatman #1 filter paper and then centrifuging at 10,000×*g* for 30 min. The supernatants were sterilized by filtering through a 0.2-μm membrane (Millipore, Bedford, MA, USA). Aliquots of 25 ml extract were stored in 50 ml sterile centrifugal tubes and lyophilized. The freeze-dried TE powder was stored at −30 °C until use.

2.2.2. Soybean

Soybean seeds were purchased from a local supermarket and were ground in a home style grinder. The ground sample was subdivided into multiple aliquots in amber bottles, flushed with nitrogen and stored at −30 °C until use. Two grams of soybean powder were mixed with 25 ml of 70% ethanol in a 50-ml centrifugal tube. The tube was shaken in a water bath at 60 °C for 2 h in dark. The ratio of soybean powder to extraction solvent was equivalent to that of homemade soymilk (*ca.* 60 soybean seeds for 100 ml soymilk). When cooled to room temperature, 1 ml of 2 M NaOH was added to the mixture and shaken at room temperature for 10 min before 0.6 ml glacial acetic acid was added. The mixture was centrifuged at 10,000×*g* for 30 min and the supernatant was collected. Pooled supernatant was adjusted to pH 6.5 and concentrated using a rotary evaporator under reduced pressure at *ca.* 50 °C and the concentrated supernatant was filtered through 0.2-μm filter membrane for sterilization. The resulting soybean extract (SBE) was lyophilized and stored at −30 °C until use.

2.3. Antibacterial activity assays

2.3.1. Agar diffusion assay

Antibacterial activities of sample extracts against four pathogens were assessed by agar diffusion assay. Pathogens were incubated in LB broth and *L. plantarum* WCFS1 in MRS broth with or without sample extracts at 37 °C for 20 h. LB agar was flooded with 550 μl of bacterial suspension to give confluent colonies. The inocula were set between 1 and 5×10^6 CFU/ml. One hundred and sixty microliters of a sample extract solution were added into an Oxford cup (a stainless cylinder with an outer diameter of 7.8 ± 0.1 mm, inner diameter of 6.0 ± 0.1 mm, and height of 10.0 ± 0.1 mm) placed on the agar surface. Plates were incubated at 37 °C for 7–9 h under aerobic conditions depending on the growth rate of each microorganism. The diameter of the inhibition zone around the cup (including that of the cup) was then measured and recorded. Zones with a diameter less than 9.0 mm were considered as "no inhibition". Each extract solution was prepared at its maximum concentration in sodium phosphate buffer (pH 6.5) (GTE: 56 mg/ml; BTE: 120 mg/ml; SBE: 48 mg/ml) and was then serially diluted (twofold). Penicillin G solutions at varying concentrations (max. 5 mg/ml) were also prepared in phosphate buffer and tested against all bacteria as a positive control. All measurements were performed at least in triplicate.

2.3.2. Broth dilution assay

Antibacterial activity was also determined using a macro-broth dilution method. Pathogens and *L. plantarum* WCFS1 were grown in growth media containing varying concentrations of sample extracts at 37°C for 20 h and bacteria cultured in growth media without sample extract served as the control. Bacterial cell population was recorded by plate count method before the start of the incubation and shortly after it was finished. Each PPE was prepared at its maximum concentration (green tea extract: 28 mg/ml; black tea extract: 60 mg/ml; soybean extract: 24 mg/ml) and was then serially diluted (twofold).

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