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Metabolites produced during the growth of probiotics in cocoa supplementation and the limited role of cocoa in host-enteric bacterial pathogen interactions

Mengfei Peng [a], Uma Aryal [b], Bruce Cooper [c], Debabrata Biswas [a, d, *]

[a] Biological Sciences Program, Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, United States
[b] Department of Biochemistry, Purdue University, IN 47907, United States
[c] Bindley Bioscience Center, Purdue University, IN 47907, United States
[d] Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742, United States

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ABSTRACT

Cocoa contains various compounds that can significantly affect the growth of a broad range of bacteria, and have multiple human health-promoting properties. In this study, the effects of cocoa powder on the growth of *Lactobacillus*, common milk resident bacteria, and three major foodborne enteric bacterial pathogens; enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes*, were investigated *in vitro*. Significant ($p < 0.05$) growth stimulation on beneficial bacteria including *Lactobacillus* and other resident bacteria in milk was observed in the presence of 3% cocoa powder. In contrast, growth of three foodborne enteric pathogens was significantly ($p < 0.05$) inhibited within 9 h, but no stimulation was found with longer incubation. In addition, cocoa powder significantly ($p < 0.05$) inhibited adhesion to and invasion of INT407 cells by these bacterial pathogens in a dose dependent manner. These results suggest that addition of cocoa into dairy products could improve the beneficial effect of probiotics by stimulating their growth, without raising the risk of cross-contamination with enteric pathogens.

1. Introduction

Human gut flora safeguards our intestine by inducing gut innate and adaptive immunity, and preventing the growth of pathogens by competitive exclusion (Guarner & Malagelada, 2003). Multiple normal bacterial floras in human gut are also resident bacteria in milk and other dairy products (Nueno-Palop & Narbad, 2010), which not only play important roles in dairy industry, but also act essentially by maintaining and strengthening human gut health (Sears, 2005). *Lactobacillus* have consistently been shown to be beneficial to human health by improving intestinal microbial balance, activating the immune system, and increasing volatile fatty acids, triggering their anti-pathogenic activities (Matsumoto et al., 2010). They produce various bio-active molecules that help to reduce postoperative infections, which have already been applied in some surgical situations (Kanazawa et al., 2005). According to the recent study, *Bacillus subtilis* is important in the development of gut-associated lymphoid system (GALT) due to their immune–-stimulatory activity (Huang, La Ragione, Nunez, & Cutting, 2008). Meanwhile, *Enterococcus faecalis* and *Streptococcus thermophilus* are shown to prevent the colonization of pathogenic bacteria in human gut by competing for binding sites and nutrients (Nueno-Palop & Narbad, 2010). However, it has been tested that the composition of gut microbiota and its functions are affected by daily intake of various foods and beverages (Martin et al., 2010), and are under attack by foodborne enteric pathogens. Among these pathogens, EHEC, *Salmonella*, and *Listeria* have drawn the most attention (CDC, 2013; Cummings et al., 2012; Dussurget, 2008; Teunis, Ogden, & Strachan, 2008). The human-enteric bacterial pathogens interaction and their infection process is usually initiated by intestinal epithelial cell adhesion and following by cell invasion through site-specific ligands (Ahn, Kim, Jung, & Biswas, 2014). Infections with these foodborne enteric pathogens and their severity are also highly influenced by normal gut microbiota, and the immunity of the host. In general, normal microflora

* Corresponding author. Department of Animal and Avian Sciences, Center for Food Safety and Security Systems, University of Maryland, College Park, MD 20742, United States. Tel.: +1 301 405 3791; fax: +1 301 405 7980.
E-mail address: dbiswas@umd.edu (D. Biswas).

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colonization in human's gut is effective in competitively preventing foreign bacterial pathogens from attachment (Steinhoff, 2005). As a consequence, there is an increasing interest in the use of diet especially prebiotics to modulate the composition of the colonic beneficial microflora for health-promoting effects such as immunostimulation and inhibition of pathogen while diminishing the negative effects which include carcinogen production and infection (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004).

Cocoa is a rich source of various polyphenols, including flavanols and procyanidins, which are free radical cleaning molecules with strongly antioxidant activity and anti-inflammatory potential (Bubonja-Sonje, Giacometti, & Abram, 2011; Cai, Luo, Sun, & Corke, 2004; Gu, House, Wu, Ou, & Prior, 2006; Perez-Berezo, Franch, Castellote, Castell, & Perez-Cano, 2012). The compounds from cocoa also have been shown to have numerous health-promoting properties such as reducing blood pressure, increasing the formation of endothelial nitric oxide, and promoting vasodilation (Ried, Sullivan, Fakler, Frank, & Stocks, 2012). Previous reports found that *Lactobacillus* strains were one of the predominating bacterial species of cocoa bean fermentations (Lefeber, Janssens, Camu, & De Vuyst, 2010; Papalexandratou, Camu, Falony, & De Vuyst, 2011), and there is also evidence that cocoa components like flavanols, multiple carbohydrates, and dietary fibers that reach the large intestine may have prebiotic-like benefits by promoting the growth of select beneficial gut microflora (Massot-Cladera, Perez-Berezo, Franch, Castell, & Perez-Cano, 2012; Tzounis et al., 2011). In addition, novel films of ethylene–vinyl alcohol copolymer containing flavonoid-rich cocoa have been developed and demonstrated to have an antimicrobial effect against several pathogens (Calatayud et al., 2013). However, no systematic research has evaluated the effects and application of cocoa on both probiotic benefits and foodborne pathogen cross-contamination.

The aim of this work is *in vitro* assessment of the effects of cocoa on human health from two folds: first, investigating the promotive effect on growth of beneficial bacteria including four strains of *Lactobacillus* and three resident bacteria in milk, and meanwhile detecting bio-active components in cocoa for explaining the noted growth promotion; second, assessing the effect of cocoa on growth as well as host cell interactions of foodborne enteric pathogens. The findings of this research will provide insight into the beneficial properties of cocoa on human health based on gut microbes.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Four *Lactobacillus* strains, *Lactobacillus casei* (ATCC334), *L. rhamnosus* (ATCC11443), *L. plantarum* (ATCC39542) (gift from Dr. John A. Lindquist, University of Wisconsin Madison), and *L. acidophilus* (ATCC4356) were used as probiotics in this study. *Lactobacillus* strains were grown on de Man-Rogosa-Sharpe (MRS) agar at 37 °C overnight in the presence of 5% CO_2 (Forma™ Scientific CO_2 water jacketed incubator, Thermo Scientific, Massachusetts, USA). Three resident bacterial strains including *B. subtilis* (PIC 620), *E. faecalis* (PIC 522A), and *Streptococcus Salivarius* subsp. *thermophilus* (ATCC19258) were also tested in this study. *B. subtilis*, *E. faecalis*, and *S. thermophilus* were grown on Brain–heart infusion (BHI) agar at 37 °C overnight under aerobic conditions (Thermo Scientific MAXQ 4450, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Enterohemorrhagic *Escherichia coli* EDL933 (ATCC700927) (gift from Dr. Michael Doyle, University of Georgia), *Salmonella enterica* serovar Typhimurium LT2 (ATCC19585), and *Listeria monocytogenes* LM2 (ATCC19115) (gift from Dr. Steven Ricke, University of Arkansas), were used in this study. EHEC, *S.* Typhimurium, were grown on Luria–Bertani (LB) agar, and *L. monocytogenes* was grown on BHI agar, at 37 °C overnight (18 h) under aerobic conditions. All agar media were purchased from Gibco, Grand Island, NY.

2.2. Cocoa powder preparation

Commercial, non-alkali treated cocoa was purchased at the local supermarket. Cocoa powder was defatted with hexane for 18 h and the residual hexane was evaporated from the cocoa powder before use. The defatted cocoa powder was stored at 4 °C and sterilized 2 h under ultraviolet light before experimental use. Cocoa powder was mixed in MRS broth. The cocoa-MRS solution was used for HPLC-MS analysis. The highest 3% (w/v) cocoa concentration treatment was selected based on the commercial content of cocoa in cocoa-containing drinks.

2.3. Viability assay of Lactobacillus strains, milk resident bacteria, and foodborne bacterial pathogens

L. casei, *L. rhamnosus*, *L. plantarum*, *and L. acidophilus* on MRS agar plates were collected in phosphate buffer saline (PBS) and the optical density (OD) of the bacterial suspension was adjusted by PBS to an absorbance value of 0.2 at 600 nm which contains 10^7 colony formation unit (CFU) per mL bacterial cells using a LAMBDA BIO/BIO + spectrophotometer (PerkinElmer, Beaconsfield, UK). A 400 μL aliquot of the bacterial suspension was added into either 3.6 mL MRS broth, whole milk, or skim milk in the presence or absence of 3% (w/v) cocoa powder in sterilized culture tube (Thermo Fisher Scientific Inc.) and incubated for different time points (0, 24, 48 and 72 h) at 37 °C under aerobic conditions with 5% CO_2 (Forma™ Scientific CO_2 water jacketed incubator). Serial dilutions were performed in PBS after each incubation period, followed by plating on MRS agar in triplicate. Bacterial CFUs on plates were counted after 24 h incubation and transformed into log values, and results were expressed as average number of CFU in log-scale. *B. subtilis*, *E. faecalis*, and *S. thermophilus* were grown and plated on BHI agar, and their viability in whole milk was investigated following the method described above. *L. acidophilus* was also recognized as well as resident bacteria in milk. Likewise, bacterial suspensions of EHEC, *S.* Typhimurium, and *L. monocytogenes* in PBS were adjusted to absorbance value of 0.100 at 600 nm to get 10^7 CFU/mL. LB broths with and without 3% (w/v) cocoa powder was used as media for growth comparison on different time periods (0, 3, 6, 9, 12, 24, 48, and 72 h). After incubation, foodborne pathogens were plated and counted on strain-specific agar plates (MacConkey agar for EHEC, Xylose Lysine Deoxycholate agar for *S.* Typhimurium, and Oxford *Listeria* agar base for *L. monocytogenes*) overnight at 37 °C under aerobic conditions (Thermo Scientific MAXQ 4450).

2.4. Cell adhesion and invasion assay

Human intestinal INT407 cells (ATCC CCL-6) were cultured at standard condition (37 °C, 5%CO_2, 95% humidity) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/mL of penicillin, and 100 U/mL of streptomycin. The INT-407 cells were seeded at 2×10^6 cells/mL into 24-well plate and cultured at standard condition mentioned above up to more than 85% confluence. The semi-confluent cultures were washed three times with phosphate-buffered saline (PBS) and immersed in serum-free DMEM for cell adhesion and invasion assay. Cell adhesion and invasion assay was performed in accordance with the method described previously with slight modification (Peng, Bitsko, & Biswas, 2015; Salaheen, White, Bequette, & Biswas, 2014). Briefly, the INT407 cells grown in 24-well plate with 800 μL DMEM were pre-treated with 100 μL