Effect of *Escherichia coli* and *Lactobacillus casei* on Luteolin Found in Simulated Human Digestion System

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Abstract   This study was conducted to investigate the effects of *in vitro* human digestion and enterobacteria (*Escherichia coli* and *Lactobacillus casei*) on the digestibility and structure of luteolin. Luteolin was passed through an *in vitro* digestion system that simulates the composition of the human mouth, stomach, small intestine, and large intestine and contains enterobacteria. The luteolin content was not altered by mouth or stomach digestion, but it was decreased by small intestine digestion. Large intestine digestion by enterobacteria also decreased the luteolin content; *L. casei* reduced the luteolin content more than *E. coli*. Moreover, digestion in the large intestine by a combination of *E. coli* and *L. casei* reduced the luteolin content more than digestion by individual enterobacteria. This study will provide insight into how enterobacteria influence the digestibility and structure of phytochemicals.

Keywords: In vitro human digestion, luteolin, enterobacteria, escherichia coli, lactobacillus casei

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1. Introduction

Phytochemicals are well known for their various pharmacological properties, such as antioxidant and disease preventive/protective effects. Polyphenol luteolin (a yellow flavone) is a common dietary form of luteolin, which is present in various fruits, herbs, and vegetables (Lin, et al., 2008; Mencherini, et al., 2007). Luteolin has been shown to have stronger antioxidant activity and lower potential prooxidant activity than many common flavonoids, such as quercetin and myricetin, and to show potential health benefits in humans (Seelinger, et al., 2008). Numerous studies have shown that luteolin exhibits a number of health-promoting functions, including anticancer (Lee, et al., 2001; Ong, et al., 2010), anti-inflammatory (Seelinger et al., 2008; Xagorari et al., 2001), and cardioprotective (Liao, et al., 2011) properties. Therefore, luteolin is regarded as one of the most important bioactive phytochemicals, with potential applications in a wide range of pharmaceutical, nutraceutical, and functional food products.

In nutritional studies in both animals and humans, *in vivo* feeding methods usually provide the most precise results. However, they are time-intensive and expensive, which is why much effort has been dedicated to the development of *in vitro* processes (Boisen & Eggum, 1991). *In vitro* human digestion provides a useful alternative to *in vivo* models for rapidly screening food materials. The ideal *in vitro* human digestion model would provide accurate results in a short time and could thus serve as a tool for rapid screening of foods and delivery systems with different compositions and structures (Coles, et al., 2005; Fuller, 1991). A review by Hur et al. (2011) (S. J. Hur, Lim, et al., 2011) reported that during the last few decades, researchers have utilized a number of *in vitro* human digestion models to test the structural and chemical variation in different foods under simulated gastrointestinal conditions, although the reliability of these models has not yet been widely accepted. However, the effect of enterobacteria on phytochemicals during *in vitro* human digestion has not yet been evaluated. Therefore, development of new technologies in search for form changes of phytochemical by another factor is opportunities for the research database.

2. Materials and Methods

2.1. Materials

Bicarbonate, potassium thiocyanate, sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, calcium chloride, ammonium chloride, urea, a-amylase, uric acid, mucin, bovine serum albumin, pepsin, pancreatin, lipase, bile salt extraction were purchased from Sigmae Aldrich chemical company (St Louis, MO, USA). All other reagents were of the highest grade commercially available.

2.2. *In vitro* Human Digestion Model

A digestion model that simulates the mouth, stomach, and intestine was used in this research, modified from

those described by previous studies (Versantvoort, et al., 2005; Hur et al., 2009; Hur et al., 2011). To simulate digestion in the mouth, a 5 mL sample of luteolin in DMSO was mixed with 5 mL of saliva fluid (pH 6.8) and stirred for 5 min. Approximately 10 mL of simulated gastric fluid (pH 1.3) was added and the mixture was stirred with a magnetic stirrer for 2 h. Finally, 10 mL of duodenal juice (pH 8.1) and 5 mL of bile juice (pH 8.2) were added and the mixture was stirred for 2 h. All digestive juices were heated to 37°C. The composition of the simulated saliva, gastric, duodenal, and bile fluids are listed in Table 1. During *in vitro* simulation of human digestion, the large intestine step requires enterobacteria such as *E. coli* and *L. casei*. Following the small intestine step, 35 mL of *E. coli* and *L. casei* were applied to the sample and incubated for 4 h at 37°C. A schematic diagram of the *in vitro* model of human digestion of luteolin using enterobacteria is shown in Figure 1.

Table 1. Constituents and concentrations of the various synthetic juices used in the *in vitro* human digestion model

	Saliva	Gastric juice	Duodenal juice	Bile juice
Organic and inorganic components	1.7 mL NaCl[1] (175.3 g/L) [2]	6.5 mL HCl (37 g/L)	6.3 mL KCl (89.6 g/L)	68.3 mL NaHCO$_3$ (84.7 g/L)
	8 mL urea (25 g/ L)	18 mL CaCl$_2$.2H$_2$O (22.2 g/L)	9 mL CaCl$_2$ · 2H$_2$O (22.2 g/L)	10 mL CaCl$_2$ · 2H$_2$O (22.2 g/L)
	15 mg uric acid	1 g bovine serum albumin	1 g bovine serum albumin	1.8 g bovine serum albumin
				30 g bile
Enzymes	290 mg α-amylase	2.5 g pepsin	9 g pancreatin	
	25 mg mucin	3 g mucin	1.5 g lipase	
pH	6.8 ± 0.2	1.50 ± 0.02	8.0 ± 0.2	7.0 ± 0.2

[1] These numbers show the concentration of the chemicals used to make the digestive juices.
[2] The numbers in parentheses show the concentrations of inorganic or organic components per liter of distilled water.
After mixing all ingredients (inorganic components, organic components, and enzymes), the volume was increased to 500 mL with distilled water. If necessary, the pH of the juices was adjusted to the appropriate value.
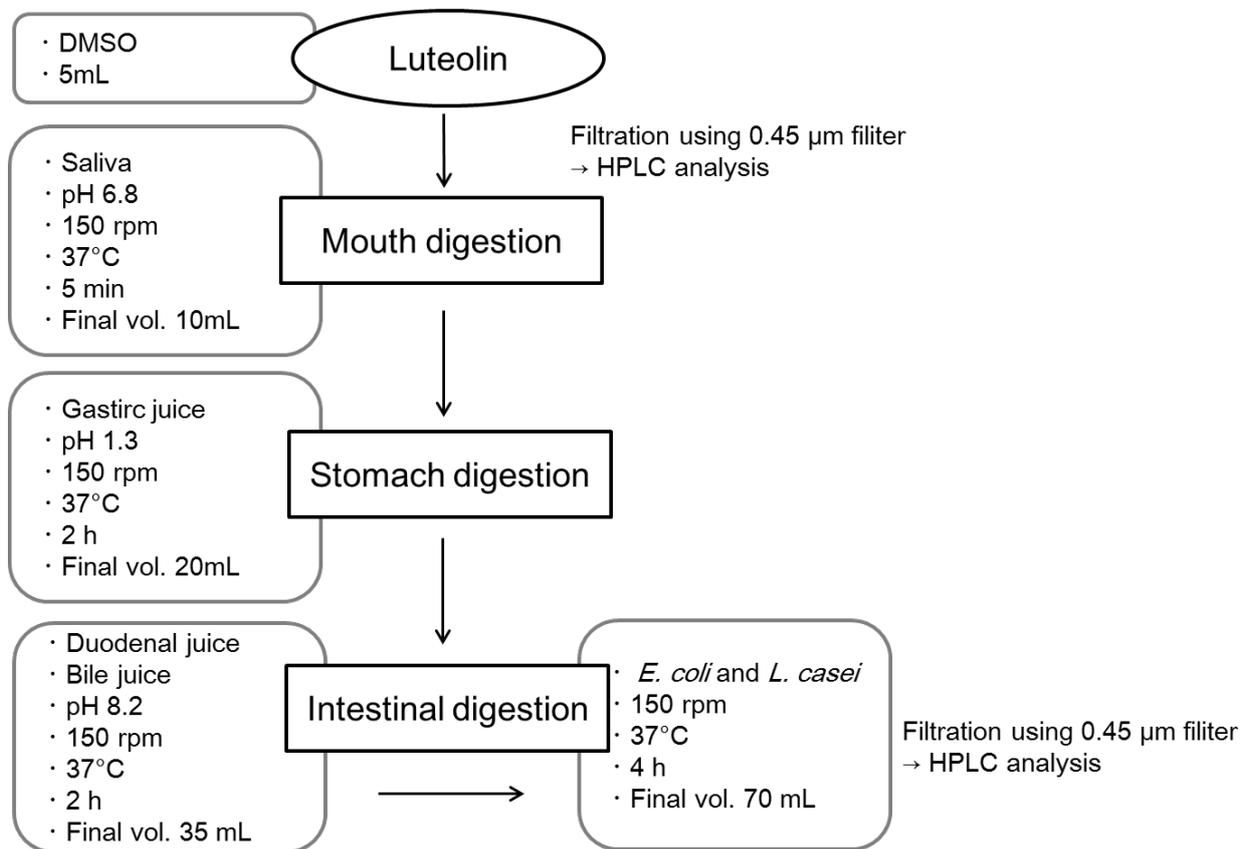

Figure 1. Schematic diagram of the *in vitro* human digestion of luteolin and subsequent chemical analysis

2.3. Preparation of Microorganisms

E. coli was obtained from the American Type Culture Collection (ATCC), and *L. casei* MCL was isolated from feces collected from healthy adults. The *E. coli* and *L. casei* were stored at -80°C until they were used. The *E. coli* was cultured in Luria-Bertani (LB) medium (Difco™ LB broth, Miller, MD, USA) and the *L. casei* was cultured in MRS medium (Difco™ Lactobacilli MRS Broth, Miller, MD, USA) in a shaker-incubator at 37°C and 150 rpm.

After incubation, the final colony counts for *E. coli* and *L. casei* were in the range of log 10^8–10^{10}.

2.4. Measurement of Luteolin by HPLC

Luteolin, digested luteolin, and luteolin digested by intestinal microorganisms were analyzed using high-performance liquid chromatography (HPLC, HP Agilent 1100, Hewlett Packard Co) on a Fortis H$_2$O column (250 mm × 4.6 mm, 3 μm) using a water : tetrahydrofuran: trifluoroacetic acid gradient (97.9 : 2 : 0.1, v/v/v) and

acetonitrile at a flow rate of 1.2 mL/min. The volume of sample injected for analysis was 20 µL, and the detection wavelength was set at 350 nm. All solutions were passed through a 0.45-µm Whatman membrane filter before injection onto the HPLC column.

3. Results and Discussion

The results showed the changes of luteolin contents at different concentrations (0.5, 1.0, and 2.0 mg/mL) during *in vitro* human digestion (Table 2). Changes in luteolin content were not observed following mouth or stomach digestion (data not shown), but luteolin content was reduced after small intestine and large intestine digestion. Thus, luteolin is stable under the digestion conditions found in the mouth and stomach, but is unstable under the digestion conditions found in the small intestine. A possible mechanism for the changes in luteolin content observed during *in vitro* human digestion is pH fluctuation. Boyer et al. (2005) found that flavonoids are stable at lower storage pH and less stable at higher pH (Boyer, et al., 2005). Bermudez-Soto et al. (2007) also reported that polyphenols are largely stable during gastric digestion (under acidic conditions), but that they are quite sensitive to mildly alkaline conditions, as found in the small intestine. In the model of digestion used in this study, the pH shifts dramatically between the stomach and the small intestine, from pH 1.5 to pH 7.5, mainly because bile salt has a higher pH. This change in pH is the primary factor involved in the irreversible breakdown of luteolin. Therefore, we hypothesize that *in vitro* digestion in the small intestine of humans may affect the structure of luteolin.
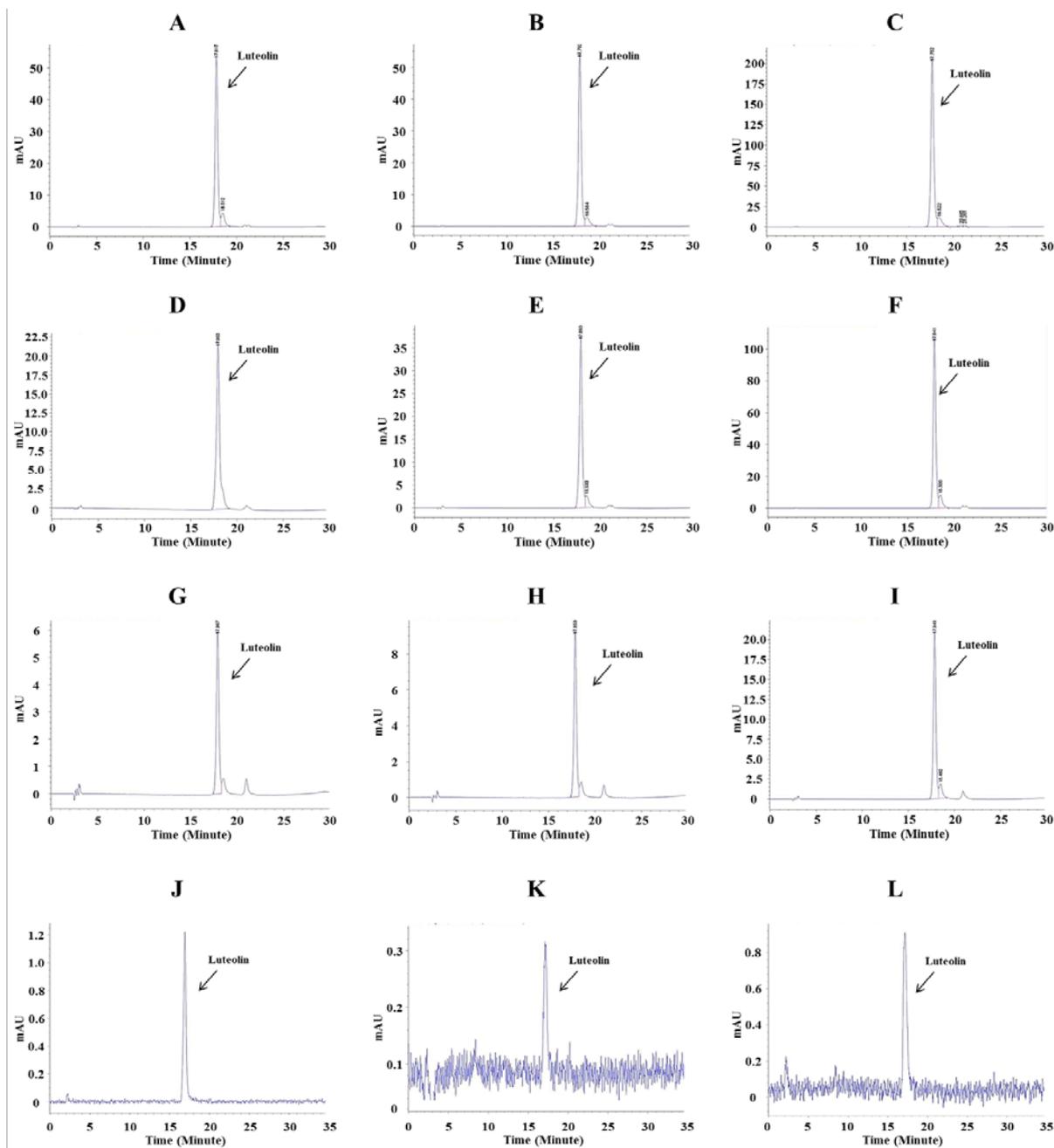

Figure 2. Effect of *in vitro* human digestion and enterobacteria on the digestion/absorption rate of luteolin. A; 0.5, B; 1.0, C; 2.0 mg/mL of undigested luteolin. D; 0.5, E; 1.0, F; 2.0 mg/mL of digested luteolin. Luteolin following *in vitro* digestion with *E. coli* (G; 1.0, H; 2.0 mg/mL). Luteolin following *in vitro* digestion with *L. casei* (I; 1.0, J; 2.0 mg/mL), Luteolin following *in vitro* digestion with mixed *E. coli* and *L. casei* (K; 1.0, L; 2.0 mg/mL)
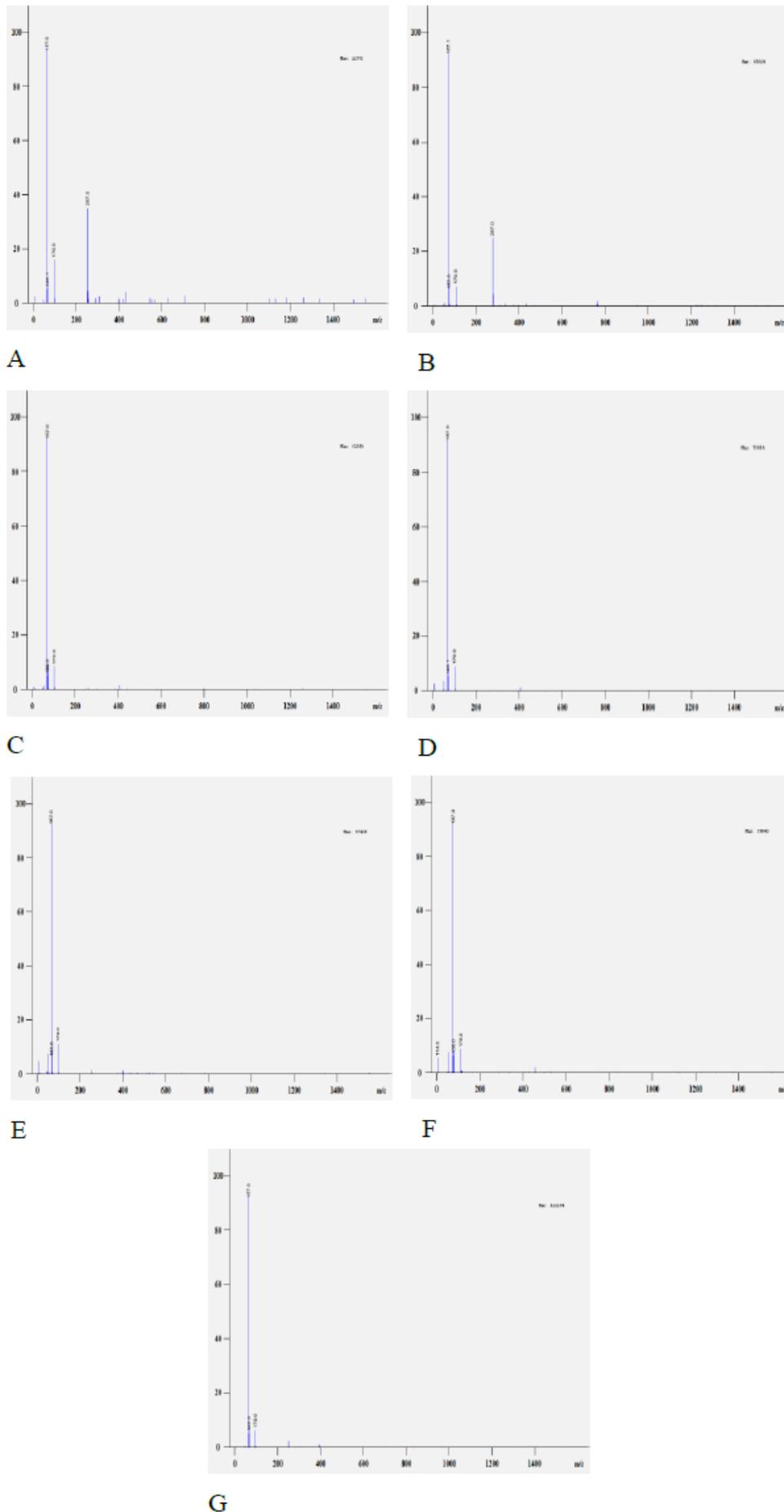
Figure 3. Effect of *in vitro* human digestion and enterobacteria on the mass spectrum of luteolin. A: luteolin, B: luteolin following mouth digestion, C: luteolin following stomach digestion, D: luteolin following small intestine digestion, E: luteolin following large intestine digestion with *E. coli*, F: luteolin following large intestine digestion with *L. casei*, G: luteolin following large intestine digestion with *E. coli* and *L. casei*

Table 2. Effect of *in vitro* human digestion and enterobacteria on the digestibility and structure of luteolin

Luteolin digestibility (absorption rate)			
Before digestion (original content of luteolin)	After *in vitro* human digestion		
mg/mL			
0.5	0.28 ± 0.07		
1.0	0.41 ± 0.02		
2.0	0.36 ± 0.11		

Level of luteolin (%)			
Luteolin concentration	0.5	1.0	2.0
	%		
Before digestion	100	100	100
After small intestine digestion	69.73	54.84	34.99
In vitro digestion + *E. coli*	-	5.12	3.10
In vitro digestion + *L. casei*	-	17.56	0.50
In vitro digestion + *E. coli* + *L. casei*	-	0.28	0.30

E. coli is known to be found in the large intestines of humans and animals, and to aid in the processes of digestion and absorption. *Lactobacillus* spp. can be found in materials isolated from plants and the gastrointestinal tracts of humans and animals (Walter, 2008). These enterobacteria affected the luteolin content during *in vitro* digestion in humans. In this study, the luteolin content was decreased during large intestine digestion (Figure 2 G–L). This result may be due to the effects of enterobacteria (*E. coli* and *L. casei*) during large intestine digestion. Luteolin contents determined that the antagonism of microorganism or flavonoid influenced by pH. Shimoi et al. (1998) investigated the intestinal absorption of luteolin7-*o*-*β*-glucoside and luteolin in rats and humans by HPLC (Shimoi, et al., 1998). They found that luteolin 7-*o*-*β*-glucoside was absorbed after its hydrolysis to luteolin by intestinal microbes, which can hydrolyze glucosides of flavonoids. Braune et al. (2001) also reported that luteolin was first degraded to eriodictyol and then to 3-(3,4-dihydroxyphenyl) propionic acid and phloroglucinol by reduction of the double bond in the 2,3-position, which was observed following fermentation by the human intestinal tract bacterium *Eubacterium ramulus*. Our previous survey found that lactic acid bacteria possess various active enzymes such as amylase, dehydrogenases, glucosidase, phenolic acid decarboxylases, and phenol reductase (Hur et al., 2014). Luteolin contains four hydrogen bond donors and six hydrogen bond acceptors, and these hydrogen bonds could be cleaved by the hydrolysis enzymes possessed by the lactic acid bacteria used in this study. In Figure 2 (G–J), the luteolin content was higher following digestion with *E. coli* than following digestion with *L. casei,* during large intestine digestion. Thus, *L. casei* may influence the luteolin content more strongly than *E. coli* during *in vitro* human digestion. *L. casei* may have a stronger effect due to its production of lactic acid; the consequent pH fluctuations could accelerate hydrolysis of luteolin during digestion in the large intestine. Furthermore, the luteolin content was found to be lower following digestion with both *E. coli* and *L. casei* than following digestion by individual enterobacteria in the large intestine. Thus, digestion by individual enterobacteria more strongly affects the structure of luteolin than digestion by enterobacteria in combination, in the large intestine (Figure 3). This result

may be due to antagonism between *E. coli* and *L. casei* during *in vitro* human digestion. The mechanism of the antagonism remains unclear, but we hypothesize that lactic acid production by *L. casei* and the consequent pH decline might inhibit the growth of *E. coli*. Lactic acid can act on the cell membrane of *E. coli,* changing the fatty acid composition and affecting the H^+ and Na^+ ion exchange or H^+/ATPase activity of the plasma membrane. In this study, we found that the population of *E. coli* was smaller than that of *L. casei* during *in vitro* human digestion, although the initial populations were the same (data are not shown). In an earlier study, Reid et al. (1988) found that lactobacilli were coaggregated with *E. coli*. These results indicate that *L. casei* are antagonistic to *E. coli* in the gastrointestinal tract; this antagonistic activity might have influenced the digestibility and structure of luteolin during *in vitro* human digestion.

4. Conclusions

The results of *in vitro* human digestion models often differ from those of *in vivo* models because of the difficulties involved in accurately simulating the highly complex physicochemical and physiological events that occur in human digestive tracts. However, this paper is the first to investigate the relationship between enterobacteria and changes in luteolin during *in vitro* human digestion. This study will provide insight into how enterobacteria influence the digestion and structure of phytochemicals.

Acknowledgements

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