LWT - Food Science and Technology 159 (2022) 113243

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Effect of age-related *in vitro* human digestion with gut microbiota on antioxidative activity and stability of vitamins

Seung Yun Lee [a], Da Young Lee [a], Ji Hyeop Kang [a], Jae Hyeon Kim [a], Hyun Woo Kim [a], Dong Hoon Oh [a], Jae Won Jeong [a], Bum Keun Kim [b], Sun Jin Hur [a,*]

[a] Department of Animal Science and Technology, Chung-Ang University, Anseong-si, Gyeonggi-do, 17546, South Korea
[b] Research Group of Food Processing, Korea Food Research Institute, Jeonbuk, 55365, South Korea

ARTICLE INFO

Keywords:
In vitro human digestion
Gut microbiota
Vitamins
Antioxidative activity

ABSTRACT

We aimed to determine the effect of age (adults vs elderly) on the antioxidant activity, digestibility, and stability of vitamins (vitamin C, vitamin B_6, and vitamin B_{12}) during *in vitro* human digestion. Antioxidant activity and digestibility of vitamin C were not influenced by age, digestion phase, or the gut microbiota, though its stability decreased in the presence of the gut microbiota. The activities of vitamins B_6 and B_{12} differed by age, digestion phase, and the presence of the gut microbiota. Notably, the antioxidant activity and stability of vitamin B_6 were reduced under low pH and gut microbiota, respectively. Vitamin B_{12} levels increased with age and in the presence of the gut microbiota. Together, vitamin C showed as a strong antioxidant regardless of age. Further, these findings could help develop strategies to increase the bioavailability of the vitamin B_6 and B_{12} in the human body by accounting for age and human digestive conditions.

1. Introduction

Since interest in geriatric health is rapidly growing in an aging society, it is important to promote the intake of nutrients that are deficient in the elderly. Among nutrients, water-soluble vitamins must be consumed from food sources such as fruits, meat, plants, and fish, because these vitamins are not stored in the human body (Gropper & Smith, 2012).

Water-soluble vitamins include vitamin C (ascorbic acid) and the vitamin B complex, including thiamine, riboflavin, niacin, carnitine, pantothenic acid, pyridoxine, biotin, folate, and cobalamin. Water-soluble vitamins are organic compounds not synthesized in the human body except for some endogenous synthesis of niacin and must be gained as trace nutrients from exogenous sources (Said & Mohammed, 2006). All water-soluble vitamins play important roles in metabolism-related pathways, including those pertaining to lipids, proteins, and nucleic acids, and they are responsible for the synthesis of critical components (DNA, RNA, ATP, hemoglobin, and red blood cells) in the human body. For this reason, a deficiency of water-soluble vitamins is implicated in risks to human health, leading to various clinical disorders such as cardiovascular problems, colorectal cancer, intestinal diseases, scurvy, neuropathy, and malabsorption (Kjeldgaard, Cohn, Casey, Hill, & Ingmer, 2012; Said & Mohammed, 2006). However, elderly people often have a poor diet due to physiological factors including gastrointestinal dysfunction, oral problems, and physical disability that prevents them from feeding themselves (Rémond et al., 2015); any of these issues can produce a deficiency in water-soluble vitamins. In the elderly, deficiency of vitamin B_6 and vitamin B_{12} often occurs and is implicated in the risk of cardiovascular disease and megaloblastic anemia, respectively (Rémond et al., 2015). In addition, although diseases including scurvy due to vitamin C deficiency are rare, intake of food rich in vitamin C is important for maintaining health in the elderly (Chernoff, 2016). Previous studies have reported methods of quantitative analysis and factors affecting intestinal absorption of water-soluble vitamins, as well as influence or intake status by age for water-soluble vitamins (Bäurer, Guo, Polnick, & Lämmerhofer, 2019).

However, there are no studies determining the differences in the antioxidant activity and digestibility of water-soluble vitamins by age (adults vs. elderly), evaluated through *in vitro* digestion. This study aimed to determine the impact of the bioactivity, digestibility and stability of the water-soluble vitamins C, B_6, and B_{12} depends on age related human digestion. This study will indicate whether certain factors cause changes in the properties of vitamins, and the results will help to develop future functional foods containing vitamins in a way that allows

* Corresponding author.
E-mail address: hursj@cau.ac.kr (S.J. Hur).

https://doi.org/10.1016/j.lwt.2022.113243
Received 21 July 2021; Received in revised form 8 February 2022; Accepted 12 February 2022
Available online 14 February 2022

the optimum beneficial effect of the ingested vitamin. The aim of this study was to confirm that changes in water-soluble vitamins in the body depend on age during digestion process; to accomplish this, we compared changes in the antioxidant activities, digestibility and stability of water-soluble vitamins during *in vitro* human digestion using gut microbiota from adults and elderly individuals.

2. Materials and methods

2.1. Materials

All chemicals and reagents used were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥98%), potassium persulfate (≥99%), methanol (99.8%), ferric chloride (97%), ferrozine (97%), potassium ferricyanide (99%), butylated hydroxytoluene, malondialdehyde tetra-butylammonium salt (≥97%), α-amylase (~50 U/mg), pepsin (≥250 units/mg), pancreatin (4 × USP/g), lipase (100–500 units/mg), uric acid (≥99%), mucin, bovine serum albumin (≥98%), bile salt, vitamin C (L-ascorbic acid, 47863), vitamin B_6 (pyridoxine, ≥98%), and vitamin B_{12} (cyanocobalamin, ≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (99%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and 2-thiobarbituric acid (TBA, ≥99%) was purchased from MP Biomedicals (CA, USA). The buffer solution was purchased from Samchun Pure Chemical Co., LTD (Seoul, Republic of Korea). 0.22 μm membrane filter was purchased from Hyundai Micro Co., LTD (Seoul, Republic of Korea).

2.2. Preparation of vitamin samples

Solutions of various water-soluble vitamins, including vitamin C, vitamin B_6, and vitamin B_{12}, were prepared by dissolving the vitamins in distilled water (DW) before analysis. Briefly, the vitamins (10 mg) were dissolved using a vortex mixer in 20 mL of DW in a 50 mL tube covered with foil to prevent exposure to light. The prepared vitamins were filtered through a 0.22 μm membrane filter before analysis. The final concentration of vitamins was adjusted to be 0.5 mg/mL, according to the amount of each enzyme added during the *in vitro* digestion phase. All solutions were filtered with a 0.22 μm membrane filter before measuring their antioxidant activities or injecting them into the HPLC column.

2.3. In vitro human digestion procedure by age groups

Previous studies have reported changes in the digestibility and bioaccessibility of foods and pharmaceuticals during gastric intestinal digestion using an *in vitro* digestion model (Hur, Lee, & Lee, 2015; Hur, Lim, Decker, & McClements, 2011; Lee, Kim, Yoon, & Hur, 2018; Lee, Lee, & Hur, 2021). Unlike the previous *in vitro* digestion procedures that did not consider digestion condition by ages, the *in vitro* human digestion procedure in this study considered the *in vitro* digestion conditions depends on ages including adults and elderly individuals: motility, amount of digestive enzymes, and composition ratio of gut microbiota (Fig. 1). Additionally, for the gut microbiota involved in the large-intestine digestion, *Lactobacillus casei* (*L. casei*) and *Escherichia coli* (*E. coli*) were selected as representative microorganisms. The procedure by age groups in detail were presented in the "*2.3.2. In vitro human digestion in simulated adult gastrointestinal tract*" and "*2.3.3. In vitro human digestion in simulated elderly gastrointestinal tract*"sections.

2.3.1. Preparation of microorganisms for using large intestine digestion

The microorganisms selected was prepared to use large intestine digestion. Briefly, culture broth media of *L. casei* and *E. coli* were prepared by dissolving Lactobacilli MRS broth and Luria-Bertani (LB) broth in distilled water in accordance with adequate formula. The prepared culture media was sterilized by using autoclave (AccuResearch Korea, Inc., Seoul, Korea), and cooled the culture media down after then, *L. casei* and *E. coli* were inoculated to culture. The bacteria strains used in this research were distributed from Korean Collection for Type Cultures (Jeongeup-si, South Korea). They was incubated two times for activation at 37 °C for 12 h. The microorganisms activated were used to simulate large intestine digestion at 10^8–10^{10} CFU/mL.

2.3.2. In vitro human digestion in simulated adult gastrointestinal tract

Each of the prepared water-soluble vitamins 1 g was added to a 15 mL tube. To perform mouth phase, 1 g of each of the prepared water-soluble vitamins was mixed with 1 mL of saliva solution (pH 6.8), and then stirred at 37 °C and 150 rpm for 5 min in a shaking water bath

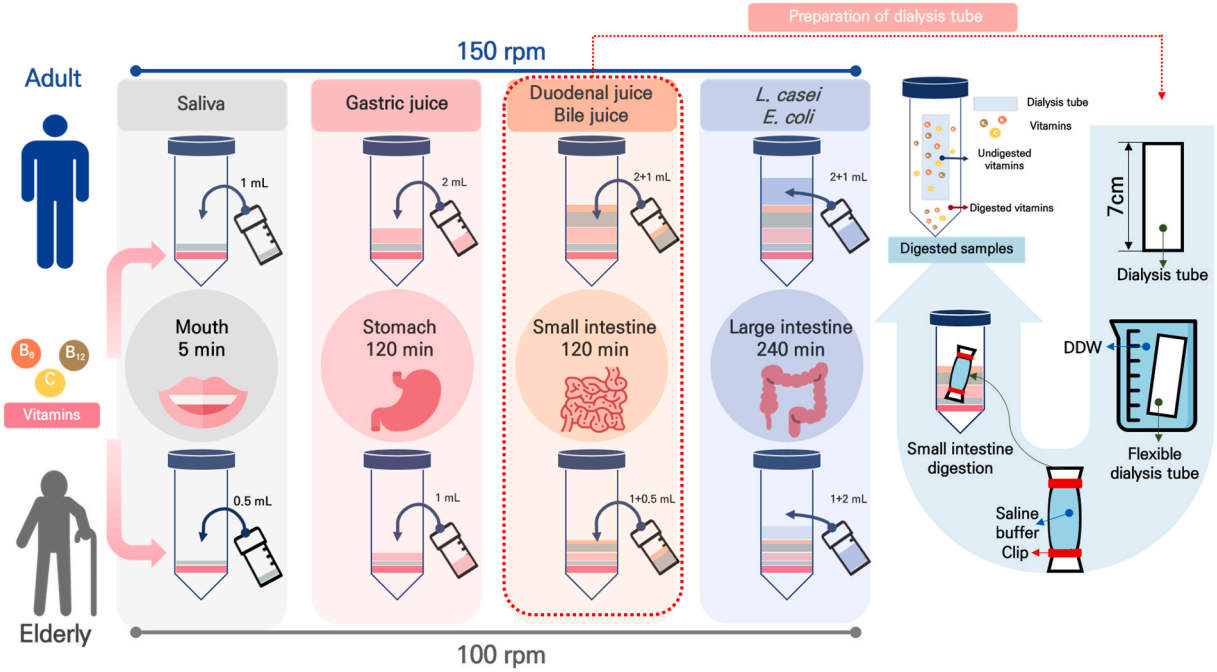


Fig. 1. Overview of the in vitro human digestion procedure for adult and elderly.

(Daihan Scientific Co. Ltd., Wonju, Korea). After the mouth digestion phase, 2 mL of gastric juice (pH 1.5) was added to each mouth-phase sample, and then stirred at 37 °C and 150 rpm for 120 min in a shaking water bath. After the stomach digestion phase, 2 mL of the duodenal juice and 1 mL of the bile juice were added to each gastric-phase sample, and then stirred at 37 °C and 150 rpm for 120 min in a shaking water bath. After the stomach digestion phase, 2 mL of *L. casei* and 1 mL of *E. coli* culture suspensions were added to the small intestine-phase sample, and then stirred at 37 °C and 150 rpm for 240 min in a shaking water bath.

2.3.3. In vitro human digestion in simulated elderly gastrointestinal tract

Each of the prepared water-soluble vitamins 1 g was added to a 15 mL tube. To perform mouth phase, 1 g of each of the prepared water-soluble vitamins was mixed with 0.5 mL of saliva solution (pH 6.8), and then stirred at 37 °C and 100 rpm for 5 min in a shaking water bath. After the mouth digestion phase, 1 mL of gastric juice (pH 1.5) was added to each mouth-phase sample, and then stirred at 37 °C and 100 rpm for 120 min in a shaking water bath. After the stomach digestion phase, 1 mL of duodenal juice and 0.5 mL of bile juice were added to the mixture of each gastric-phase sample, and then stirred at 37 °C and 100 rpm for 120 min in a shaking water bath. After the stomach digestion phase, 1 mL of *L. casei* and 2 mL of *E. coli* culture suspensions were added to the small intestine-phase sample, and then stirred at 37 °C and 100 rpm for 240 min in a shaking water bath. Distilled water was added to the enzymes to adjust their final concentration in the samples; the difference in concentration was caused by the varying amounts of digestive enzymes used in the digestion process.

2.4. Digestibility of vitamins using dialysis tube

Digestibility of vitamins was measured using dialysis tube on small intestine digestion with modification previous study (Lee, Lee, et al., 2021). Briefly, a 7-cm dialysis tube [molecular weight cutoff, 50 kDa; flat width, 34 mm; thickness, 18 μm (Membrane Filtration Products, Inc., Seguin, TX, USA)] was prepared for a 1 mL buffer solution (pH 7.00 ± 0.02) and soaked in water for a few minutes to induce flexibility. The remaining water was removed from the softened dialysis tube, and one end of the tube was closed using a clip. Then, 1 mL of buffer solution (pH 7.00 ± 0.02) was added to the softened dialysis tube, and the open side of the tube was tied using a clip. The prepared dialysis tube was used as an artificial small intestine to estimate digestion or absorption of the water-soluble vitamins in order to simulate small-intestinal digestion during *in vitro* digestion. This process can estimate that the water-soluble vitamins measured in outside dialysis tube is digested vitamins, while the water-soluble vitamins measured in inside dialysis tube is undigested vitamins. Digestibility percentage was calculated to use concentration outside dialysis tube and total concentration as outside and inside dialysis tube.

2.5. Antioxidant activities

2.5.1. ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity of the samples was determined by modifying the method described by Re et al. (1999). The ABTS solution was prepared using 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate. The solution was activated by incubating it for 18 h in the dark. The $ABTS^{\bullet+}$ solution was diluted with methanol to adjust the absorbance to 0.70 ± 0.02 at 734 nm. Then, 10 μL of each sample was added to 990 μL of the $ABTS^{\bullet+}$ solution and mixed for 5 min. The resulting samples were measured using a Sunrise microplate reader (Tecan, Männedorf, Switzerland) at 562 nm. The control samples were methanol and $ABTS^{\bullet+}$ solutions, and the experimental samples were the samples treated with the $ABTS^{\bullet+}$ solution.

The ABTS radical cation scavenging activity was calculated as:

$$\text{ABTS radical cation scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

where A_{sample} and $A_{control}$ indicate the absorbance of the sample and control, respectively.

2.5.2. Reducing power

The reducing power of the samples was determined using the method of Oyaizu (1988). Briefly, 1 μL of each sample, 1 mL of 0.2 mol/L PBS (pH 6.6), and 1 mL of potassium ferricyanide (10 mg/mL) were added to a test tube. Reaction mixtures were incubated for 20 min at 50 °C in the dark. Subsequently, 1 mL of trichloroacetic acid solution (10 g/100 mL) was added to the mixtures. The mixtures were then centrifuged at 1008×*g* for 10 min, following which 0.8 mL of the supernatant was withdrawn from each tube and mixed with 0.8 mL of $FeCl_3$ solution (100 mg/100 mL). The absorbance of the mixture was then read at 700 nm.

2.6. Determination of vitamin concentrations by high-performance liquid chromatography (HPLC)

The measurement of digestibility and stability of vitamins (vitamin C, vitamin B6 and vitamin B12) was performed according to our previous studies with modification (Lee, Lee, & Hur, 2021; Lee, Lee, Yim, & Hur, 2020). These vitamins were analyzed using HPLC (HP Agilent 1100, Hewlett Packard Co., CA, USA) with a Fortis H_2O column (250 mm × 4.6 mm, 3 μm). Vitamin C was determined with 60% of solution A (0.05 mol/mL potassium dihydrogen phosphate in water) and 40% of solution B (acetonitrile) at a flow rate of 0.5 mL/min. The volume of the sample injected for analysis was 10 μL, and the detection wavelength was 254 nm. For vitamin B_6, solutions A (water) and B (methanol, pH 2.8) constituted the mobile phase with a flow rate of 1.4 mL/min. The HPLC elution was used following as; 70% B to 65% B until 3 min, 66% B until 7 min, 70% B until 10 min, reconditioning with 70% B for 3 min. The volume of the sample injected for analysis was 10 μL, and the detection wavelength was set at 214 nm. For vitamin B_{12}, the mobile phase comprised solution A (water) and solution B (acetonitrile) at a flow rate of 1.0 mL/min. The HPLC elution was used following as; 0% B to 20% B until 11 min, 30% B until 19 min, 0% B until 26 min, reconditioning with 0% B for 4 min. The volume of the sample injected for analysis was 20 μL, and the detection wavelength was set at 361 nm. All solutions were filtered through a 0.22 μm Whatman membrane filter before injection into the HPLC column. Each sample refers to the compartment sample resulting from the specific *in vitro* digestion step (mouth, stomach, and small intestine) with vitamins.

2.7. Statistical analysis

The samples obtained through experiments vigorously was used with vigorously mixing before analysis and was chosen randomly to be fair representation of the total population. The data were expressed as the mean ± standard deviation and were performed in triplicate. All statistical analyses were performed using one-way analysis of variance (ANOVA) in GraphPad Prism software 5 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS software 26.0 (SPSS Inc., Chicago, IL, USA). Tukey's multiple comparison test was used to determine significant differences between mean values, and evaluations were based on a significance level of $p < 0.05$. The HPLC data were analyzed in triplicate to prove regression coefficient (r^2) by the calibration curve of standard using MS excel software ver. 2013 (Microsoft Co., Washington, USA).

3. Results

This study was analyzed the antioxidant activities (ABTS radical cation scavenging activity and reducing power) and amounts of water-soluble vitamins (i.e., vitamins C, B_6, B_{12}) during *in vitro* human digestion with gut microbiota from the elderly and adults to confirm the

change in the bioactivities of the vitamins, including alterations in their antioxidant activity, digestibility, and stability (Figs. 2–5). In addition, this study was performed validation method including the chromatography, recovery (%) and regression coefficient (R^2) for water soluble vitamins due to the digestibility and stability in vitamins evaluated through value confirmed by HPLC (Table 1 and Fig. 4.) Standard of vitamins including vitamin C, vitamin B6, and vitamin B12 were prepared at different concentration (ranging from 0.5 to 10 mg/mL, 0.25–2.50 mg/mL and 0.25–1 mg/mL, respectively). Under these conditions, the vitamins were distinctly separated with good peak shape. The calibration curve of the vitamin C, vitamin B6, and vitamin B12 showed remarkable linearity under different the concentration with r^2 0.994, 0.998 and 0.995, respectively. The recoveries of vitamin C, vitamin B6, and vitamin B12 were 99.52%, 107.06%, and 98.15%, respectively. These results are proved that analysis condition of vitamins used in this study are possible method for evaluating digestibility and stability.

3.1. The effects of in vitro digestion with gut microbiota from adults and elderly on the bioactivity of vitamin C

Vitamin C was evaluated to confirm whether various factors, such as age, digestion phase, and gut microbiota, affected its bioactivity. This study confirmed that vitamin C was not influenced by age, since the antioxidant activities of vitamin C did not show differences depending on age. In addition, vitamin C was not affected by digestion phases, since the antioxidant activities of vitamin C did not change as the digestion phases progressed. However, the stability of vitamin C significantly decreased in the large intestine, and was slightly affected by the gut microbiota. Thus, vitamin C is a more stable antioxidant than other vitamins within the human body, disregarding external influences.

3.2. The effect of in vitro digestion with gut microbiota from adults and elderly on the bioactivity of vitamin B_6

Vitamin B_6 was confirmed to be affected by age only with respect to its digestion in the stomach and the influence of gut microbiota. Thus, in the present study, there was no effect of age on vitamin B_6, except for in relation to stomach digestion. The ABTS radical cation scavenging activity of vitamin B_6 in the elderly (71.47% ± 1.84%) was higher than that in adults (25.74% ± 1.87%) in the stomach digestion phase ($p < 0.05$), whereas there was no difference in the reducing power between adults and the elderly. Furthermore, vitamin B_6 was found to be affected by digestion phases, as the antioxidant activities and stability of vitamin B_6 were altered in the stomach or large intestine ($p < 0.05$). The reducing power of vitamin B_6 was found to increase and its stability was found to decrease after large-intestine digestion in both adults and the elderly. This result suggests that vitamin B_6 might be considered for use in acidic conditions (low pH) prevailing during stomach digestion and in the presence of gut microbiota in the large intestine in order to increase its bioactivity in the human body.

3.3. The effect of in vitro digestion with gut microbiota from adults and elderly on the bioactivity of vitamin B_{12}

In this study, the bioactivity of vitamin B_{12} changed with age, digestion phase, and gut microbiota.

As the bioactivity of vitamin B_{12} depends on age, the ABTS radical cation scavenging activity of vitamin B_{12} in adults was higher (78.47% ± 2.95%) than that of the elderly (66.66% ± 2.61%) in the small intestine phase. Vitamin B_{12} had lower antioxidant activity than vitamin C or vitamin B_6, regardless of age. High antioxidant activity is associated with a greater number of phenolic hydroxyl groups, because the structure can donate hydrogen and electrons, resulting in the stabilization of the free radical species. We speculate that this is the reason for the low antioxidant activity of vitamin B_{12}, since the number of hydroxyl groups in vitamin C and vitamin B_6 is higher than that in vitamin B_{12}. In addition, digestibility in the elderly (45.08% ± 0.12%) was significantly higher than in adults (35.96 ± 0.11%) ($p < 0.05$). Vitamin B_{12} was mainly affected by gut microbiota, since the antioxidant activity and stability of vitamin B_{12} were found to significantly increase after large-intestine digestion. Therefore, when developing products using vitamin B_{12}, it is necessary to consider that for this vitamin, the effects of gut

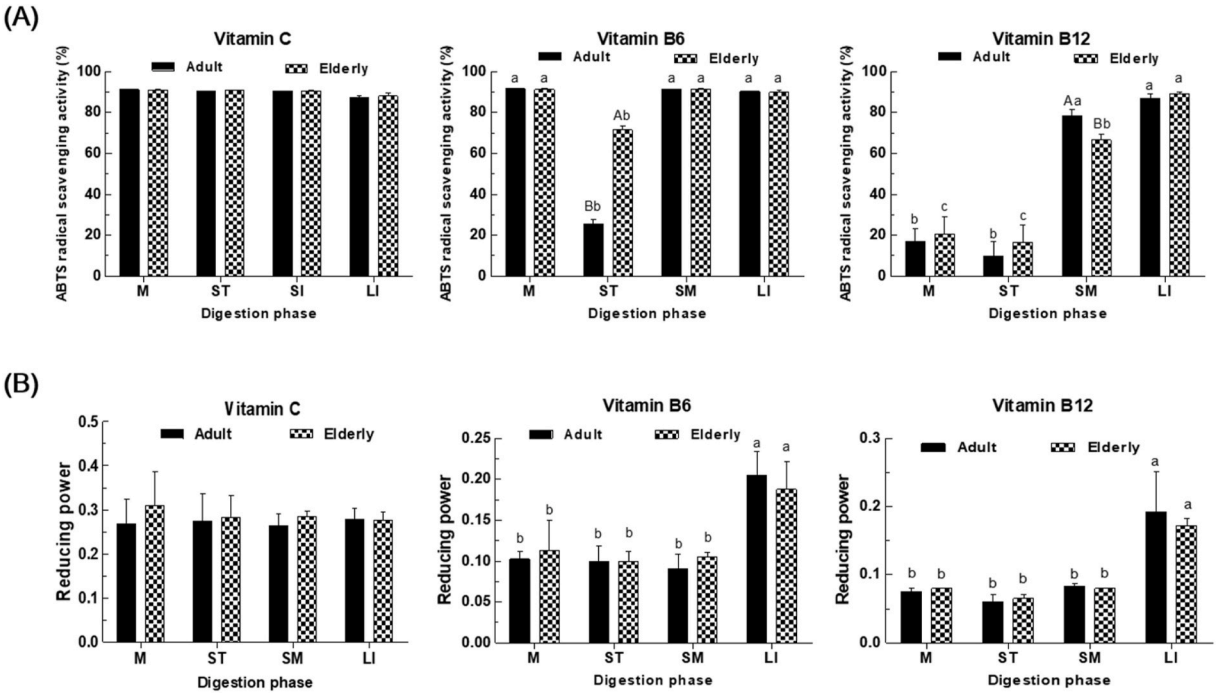


Fig. 2. (A) ABTS radical cation scavenging activity and (B) reducing power of vitamins depends on ages (adults and elderly). The data are presented as the mean values ± standard deviation (n = 3). $^{A-B}$ Means with different superscript letters differed significantly according to the ages (adult and elderly) under same digestion phase ($p < 0.05$). $^{a-c}$ Means with different superscript letters differed significantly according to the *in vitro* digestion phase ($p < 0.05$).

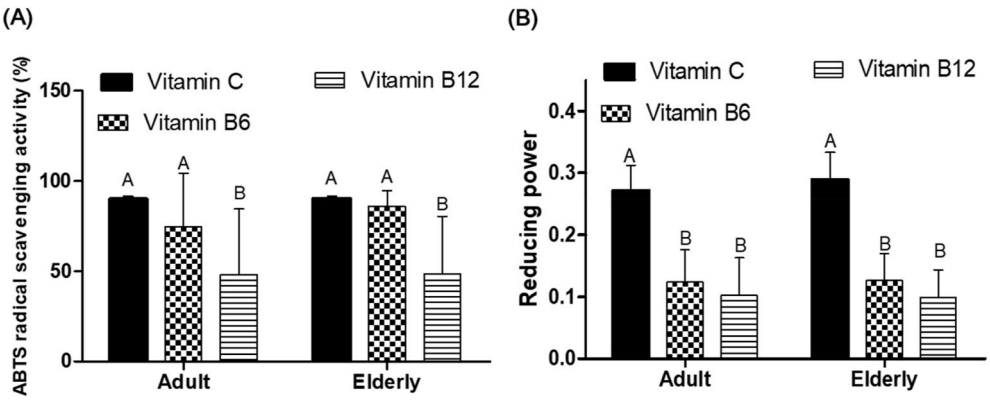


Fig. 3. (A) ABTS radical cation scavenging activity and (B) reducing power depends on vitamins. The data are presented as the mean values ± standard deviation (n = 3). $^{A-B}$ Means with different superscript letters differed significantly according to the vitamins (vitamin C, vitamin B6 and vitamin B12) ($p < 0.05$).

microbiota and the difference in optimal dosage depend on age. In addition, in many foods, the antioxidant activity of the components of the vitamin B complex declines during thermal processing. This is another aspect that should also be considered.

4. Discussion

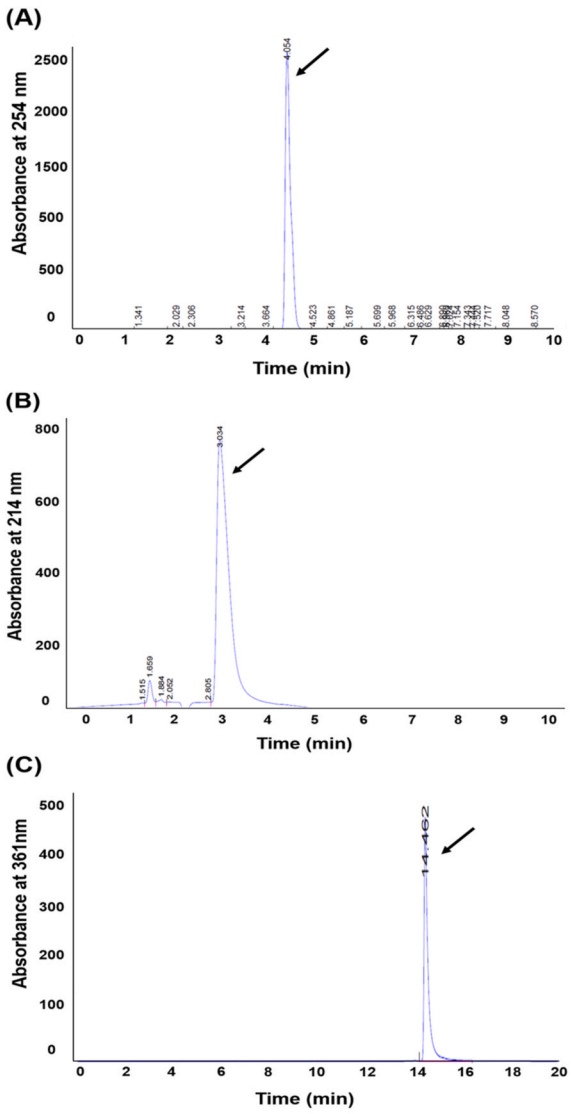
This study was compared changes in the antioxidant activities of water-soluble vitamins during *in vitro* digestion using gut microbiota from adults and elderly individuals. In contrast to our previous studies, in the present study, we used an *in vitro* human digestion model that depends on age, based on a published study regarding the significant differences in digestion conditions between adults and the elderly (Levi & Lesmes, 2014; Madsen & Graff, 2004). In Korea, the typical age of attaining legal adult status is 19 years and that for attaining the status of the elderly is over 65 years. Further, the United Nations has also advocated that the age over 65 years be designated as "old age." Digestion variables that show differences depending on age are motility, amount of digestive enzyme secretion, and composition of gut microbiota in the large intestine. Decreasing gastrointestinal motor function in the elderly is influenced by impairment of intestinal smooth muscle and of intrinsic sarcoplasmic reticulum function resulting from a decrease in intracellular Ca^{2+} levels (Bitar & Patil, 2004; Lopes et al., 2006). A previous study showed significant decrease in the secretion of digestive enzymes, including saliva, pepsin, pancreatic, chymotrypsin, and bile acid in the elderly compared to adults (Feldman, Cryer, McArthur, Huet, & Lee, 1996; Smith et al., 2013; Vellas et al., 1988). Furthermore, our previous study also confirmed significant differences in the composition of gut microbiota in the feces of adults (20–25 years old) and the elderly (over 65 years old) in Korea (Lee, Kang, et al., 2021). Adults have an approximately 3-fold higher relative abundance of *Lactobacillus* than do the elderly, whereas the elderly have a 3-fold relative abundance of *Escherichia* compared to adults (Lee, Lee, & Hur, 2021). Thus, this study was confirmed by using the *in vitro* digestion with the characteristics of adult and elderly using gut microbiota.

Water-soluble vitamins must be obtained from foods or exogenous sources because humans and other higher mammals lack the ability to synthesize these vitamins. Water-soluble vitamins are absorbed predominantly in the small intestine, whereas they were synthesized by the gut microbiota in the large intestine (Halsted, 2003; Rowland et al., 2018). This study investigated changes in the pharmacokinetics of vitamins in the human body, considering the impact of gut microbiota in the large intestine as well as the stored form of vitamins in foods, even though water-soluble vitamins are absorbed mainly during small-intestine digestion. Furthermore, this study revealed that the bioactivities of these vitamins might be affected by age, gut microbiota, and factors that occur during *in vitro* digestion phases.

Although the exact mechanisms behind the impact of age and *in vitro* digestion with gut microbiota on the bioactivities of vitamins are still unclear, some aspects were explained by pH and gut microbiota in the large intestine. First, the changing bioactivities of some vitamins may be affected by the change in pH during *in vitro* digestion. Vitamin C is more stable under acidic conditions (pH 3.4) and 39.5% less stable at pH 8.1 (Farah et al., 2020). A separate study likewise found that ascorbic acid was most stable at pH 3.5, with a small difference in stability was observed at pH 7.5 (Herbig & Renard, 2017). The highest stability was observed under acidic conditions, which can contribute to the fully protonated form of ascorbic acid at pH under the first pKa. The results of the present study also confirmed that the stability of vitamin C was higher under acidic conditions.

An early study found that solutions at pH levels 4 to 7 did not significantly degrade pyridoxine, the most stable form of vitamin B_6 (Saidi & Warthesen, 1983). Furthermore, the same authors claimed that this effect indicates that the influence of pH on vitamin B_6 degradation in foods is unclear. Another study also argued that the reasons for the strong effects of pH on vitamin B_6 may lie in the rather complex ionic equilibria exhibited by the various forms of vitamin B_6 (Snell & Haskell, 1970). However, vitamin B_6 is separated from proteins at gastric and intestinal pH because vitamin B_6 is not bound to proteins (Ball, 2005). Nagita et al. (1996) also reported that the bioaccessibility of pyridoxal 5'-phosphate, another form of vitamin B_6, is most likely affected by high pH (Nagita et al., 1996). Our results also indicate that vitamin B_6 was affected by pH during stomach digestion, since the ABTS radical cation scavenging activity of vitamin B_6 decreased after stomach digestion in both adults (25.74% ± 1.87%) and the elderly (71.47% ± 1.84%) compared with others. In particular, this result indicates that the high acidity caused by the addition of more stomach digestive enzymes in adults decreased vitamin B_6 bioactivity.

Vitamin B_{12} (cobalamin) exists in a combined form with animal protein and is mainly consumed through animal foods (Obeid et al., 2019). Dietary vitamin B_{12} bound to animal protein upon entering the stomach is affected by the acidic conditions in the stomach and by the action of pepsin, which releases the dietary vitamin B_{12} from the food protein to which it is bound. Free vitamin B_{12} is immediately bound to R-protein (heptocorrin) from parietal cells, which secrete HCl and intrinsic factors in the stomach and saliva (Seetharam & Alpers, 1982). R-proteins and gastric intrinsic factors are stable in acidic conditions, and they are known to act as carriers for transferring vitamin B_{12} to the small intestine (Tanner, Sturm, Baack, Liyanarachchi, & de la Chapelle, 2012). The essential role of heptocorrin is to protect acid-sensitive vitamin B_{12} as it is transferred into the ileum (Neale, 1990). Furthermore, vitamin B_{12} binds to intrinsic factors released by pancreatic enzymes in the small intestine. A previous study suggests that malabsorption of cobalamin in the elderly is caused by a decrease in pepsin and a lack of intrinsic factors in the cobalamin absorption system (Quadros, 2010). The result in this study showed that the ABTS radical



	Calibration range (mg/mL)	Regression coefficient (R^2)	Recovery (%)
Vitamin C	0.5 - 10	0.994	99.52
Vitamin B6	0.25 - 2.50	0.998	107.06
Vitamin B12	0.25 - 1	0.995	98.15

Fig. 4. HPLC chromatograms and validation data (regression coefficient (R^2) and recovery (%)) of vitamins. (A) vitamin C, (B) vitamin B6, and (C) vitamin B12.

cation scavenging activity of vitamin B12 in elderly (66.66% ± 2.61%) was lower than that of adults (78.47% ± 2.95%) after small intestine digestion. Thus, the age-dependent difference in antioxidant activities in the small intestine are attribute to the difference in pH due to the added amount of digestive enzymes that may be related to the protection and transfer of vitamin B_{12}. Thus, our results suggest that low pH (acidic condition) by stomach digestion influence on bioactivity of vitamin B_{12}. Thus, the pH-dependent difference in digestive enzyme secretion depends on age, and the digestion phase may contribute to the change in the bioactivity of the vitamin B_6 and B_{12}.

The gut microbiota may contribute to changes in the activities of vitamins. Numerous studies have demonstrated the inhibitory effect of vitamin C on bacterial species such a s *E. coli*, *Staphylococcus aureus*, *Helicobacter pylori*, and *Bacillus cereus*, as well as various fungi (Biswas et al., 2013; Verghese, Mathew, & David, 2017; Zhang, Wakisaka, Maeda, & Yamamoto, 1997). The proposed mechanism of action of antibacterial activity by vitamin C includes formation of hydrogen peroxide and ROS, as well as the generation of lactate and acetic acid (Kallio, Jaakkola, Mäki, Kilpeläinen, & Virtanen, 2012; Khameneh, Bazzaz, Amani, Rostami, & Vahdati-Mashhadian, 2016). The results of this study indicate that the stability of vitamin C significantly decreased during large-intestine digestion. Thus, this result suggests that vitamin C may be affected by *E. coli* involved in the large-intestine digestion.

The gut microbiota produces different vitamins of the vitamin B complex that are essential for the gut bacteria and the host (Uebanso, Shimohata, Mawatari, & Takahashi, 2020). Another study also reported that certain lactic acid bacteria are able to synthesize components of the vitamin B complex through fermentation processes (Capozzi, Russo, Dueñas, López, & Spano, 2012). An early study showed that uptake of vitamin B_{12} bound to cultures of *E. coli* inhibited the absorption of vitamin B_{12} in an animal model, and that the inhibitory effect was abolished when the bacteria were killed by heat (Booth & Heath, 1962). On the other hand, many studies have reported that vitamin B_{12} is biosynthesized by gut commensal bacteria in the colon; the complex structure of vitamin B_{12} requires various microorganisms to engage in aerobic or anaerobic pathways for its successful synthesis (Rodionov, Vitreschak, Mironov, & Gelfand, 2003; Swithers et al., 2012). Among these bacteria, *E. coli* can synthesize cobalamin via the salvage pathway, and *E. coli* has been used to generate δ-aminolevulinate as a necessary factor for vitamin B_{12} biosynthesis (Fang, Kang, & Zhang, 2017). In addition, previous studies have reported that lactic acid bacteria produce the vitamin B complex. *Lactobacillus* species identified in milk were found to produce a cobalamin-type compound (Martin et al., 2005), and the presence of vitamin B_6 in soy beverages fermented by cultures of *Lactobacillus* has been reported (Champagne, Tompkins, Buckley, & Green-Johnson, 2010). The digestibility and stability of vitamins was determined by calculating the ratio of the amount of vitamins in compartments obtained from *in vitro* digestion steps using HPLC (Fig. 5). This study showed high digestibility of vitamin B_{12} in adult and elderly (60.04% ± 0.0% and 62.69% ± 0.05%, respectively) in the large intestine in the presence of *E. coli*. This is likely due to the result of the greater ability of *E. coli*, which used in large intestine digestion step, to synthesize vitamin B12. In Fig. 5B–D, the age-dependent difference in stability was found in some digestive phases for vitamin B_6 and vitamin B_{12}, but not for vitamin C. In addition, gut microbiota may be affected by vitamin stability. In particular, the stability of vitamins C and B_6 was significantly lower in large-intestine digestion, whereas the stability of vitamin B_{12} was significantly higher in large-intestine digestion than in other phases. In addition, the phase-dependent difference in stability was not observed between adults and the elderly. *E. coli* can synthesize cobalamin via the salvage pathway and cyanocobalamin be transported by *E. coli* that bind to vitamin B_{12} through specific receptors on the outer membrane of the cell envelope (Di Masi, White, Schnaitman, & Bradbeer, 1973). Therefore, the high digestibility of vitamin B_{12} in the elderly may be affected by the proportion of *E. coli* used for the large-intestine digestion in the elderly, which was higher than that used for the large-intestine digestion in the adults. Moreover, the high stability of vitamin B_{12} may also be associated with the biosynthesis by *E. coli* in the large intestine. However, these results need to be verified by further studies.

5. Conclusions

This study was conducted to determine the changes in the antioxidant activity, digestibility, and stability of water-soluble vitamins C, B_6, and B_{12} during age-related *in vitro* human digestion with gut microbiota. The bioactivity of vitamin C was not affected by age or digestion phase, whereas gut microbiota can affect the stability of vitamin C. On the other hand, the bioactivity of the vitamin B_6, and B_{12} was influenced by age,

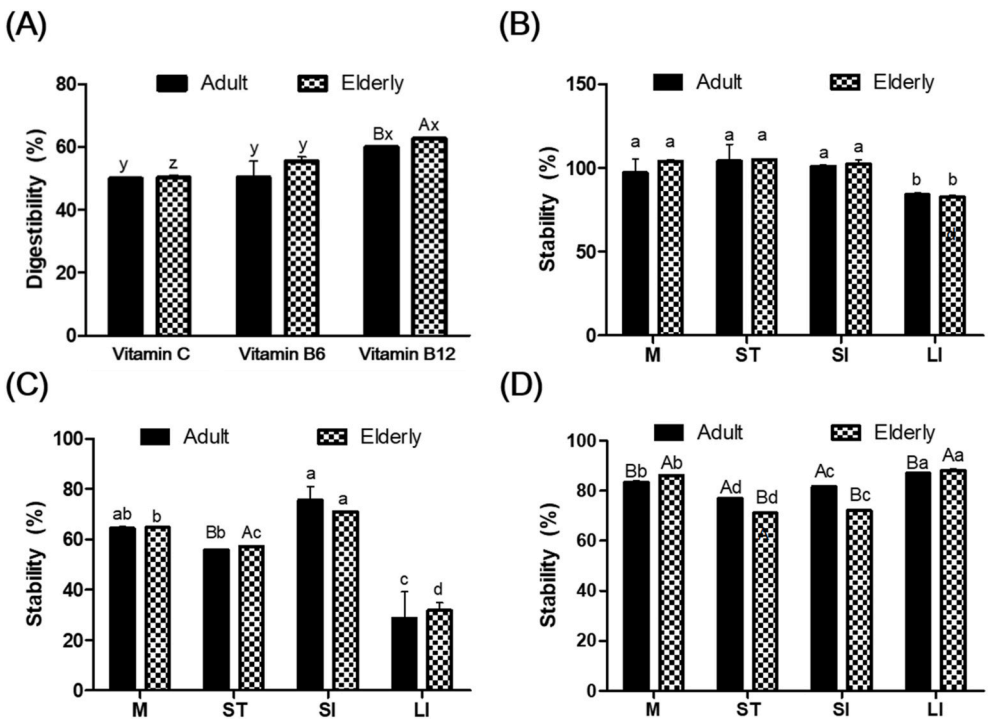


Fig. 5. (A) Digestibility and (B–D) stability of vitamins (vitamin C, vitamin B6 and vitamin B12) depends on ages. The data are presented as the mean values ± standard deviation (n = 3). $^{A-B}$ Means with different superscript letters differed significantly according to the ages (adult and elderly) under same column ($p < 0.05$). $^{x-z}$ Means with different superscript letters differed significantly according to the vitamins (vitamin C, vitamin B6 and vitamin B12) ($p < 0.05$). $^{a-c}$ Means with different superscript letters differed significantly according to the *in vitro* digestion phase ($p < 0.05$).

Table 1
Validation data for water-soluble vitamins.

	Calibration range (mg/mL)	Recovery (%)	Regression coefficient (R^2)
Vitamin C	0.5–10	99.52	0.994
Vitamin B6	0.25–2.50	107.06	0.998
Vitamin B12	0.25–1	98.15	0.995

digestion phase, and the gut microbiota. In particular, the gut microbiota may significantly contribute to changes in the activities of the vitamin B_6, and B_{12}. Taken together, our results suggest that possible strategies for the high utilization of water-soluble vitamins in the functional foods industry can be summarized as follows: (1) vitamin C is role as a strong antioxidant regardless of age, though its stability affected by the gut microbiota; (2) vitamin B_6 bioactivity improve by treating with a substance that can neutralize acidity, and by treatment with lactic acid bacteria; (3) vitamin B_{12} dosage should be regulated according to age, and bioactivity improve by utilizing gut microbiota and acid-secretion agents. Moreover, this study provides data on the changes observed in the antioxidant activities and stability of water-soluble vitamins during human digestion. However, further detailed studies need to be conducted to understand the pharmacokinetics of vitamins with respect to the changes in the activities of vitamins stored in foods and the impact of various gut microbiota.

CRediT authorship contribution statement

Seung Yun Lee: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Da Young Lee:** Investigation, Visualization, Writing – review & editing. **Ji Hyeop Kang:** Investigation, Methodology. **Jae Hyeon Kim:** Investigation, Methodology. **Hyun Woo Kim:** Investigation, Methodology. **Dong Hoon Oh:** Investigation, Methodology. **Jae Won Jeong:** Investigation, Methodology. **Bum Keun Kim:** Investigation, Validation. **Sun Jin Hur:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgement

This work has supported by the National Research Foundation of Korea(NRF) grant funded by the Korea government(MSIT)(No. 2020R1A2C100665112). This research was supported by the Chung-Ang University Research Grants in 2021.

References

Ball, G. F. (2005). *Vitamins in foods: Analysis, bioavailability, and stability*. CRC press.
Bäurer, S., Guo, W., Polnick, S., & Lämmerhofer, M. (2019). Simultaneous separation of water-and fat-soluble vitamins by selective comprehensive HILIC× RPLC (high-resolution sampling) and active solvent modulation. *Chromatographia, 82*(1), 167–180.
Biswas, S., Thomas, N., Mandal, a., Mullick, A., chandra, D., Mukherjee, S., et al. (2013). *In vitro* analysis of antibacterial activity of vitamin C alone and in combination with antibiotics on gram positive rod isolated from soil of a dumping site of Kolkata. *International Journal of Pharmacy and Biological Sciences, 3*, 101–110.
Bitar, K. N., & Patil, S. B. (2004). Aging and gastrointestinal smooth muscle. *Mechanism of Ageing and Development, 125*(12), 907–910.
Booth, C. C., & Heath, J. (1962). The effect of *E. coli* on the absorption of vitamin B12. *Gut, 3*(1), 70–73.
Capozzi, V., Russo, P., Dueñas, M. T., López, P., & Spano, G. (2012). Lactic acid bacteria producing B-group vitamins: A great potential for functional cereals products. *Applied Microbiology and Biotechnology, 96*(6), 1383–1394.
Champagne, C. P., Tompkins, T. A., Buckley, N. D., & Green-Johnson, J. M. (2010). Effect of fermentation by pure and mixed cultures of *Streptococcus thermophilus* and *Lactobacillus helveticus* on isoflavone and B-vitamin content of a fermented soy beverage. *Food Microbiology, 27*(7), 968–972.
Chernoff, R. (2016). *Elderly: Nutrition requirements*.
Di Masi, D. R., White, J. C., Schnaitman, C. A., & Bradbeer, C. (1973). Transport of vitamin B12 in *Escherichia coli*: Common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope. *Journal of Bacteriology, 115*(2), 506–513.
Fang, H., Kang, J., & Zhang, D. (2017). Microbial production of vitamin B 12: A review and future perspectives. *Microbial Cell Factories, 16*(1), 1–14.
Farah, H. S., Alhmoud, J. F., Al-Othman, A., Alqaisi, K. M., Atoom, A. M., Shadid, K., et al. (2020). Effect of pH, temperature and metal salts in different storage conditions on the stability of vitamin C content of yellow bell pepper extracted in aqueous media. *Systematic Reviews in Pharmacy, 11*(9), 661–667.

Feldman, M., Cryer, B., McArthur, K. E., Huet, B. A., & Lee, E. (1996). Effects of aging and gastritis on gastric acid and pepsin secretion in humans: A prospective study. *Gastroenterology, 110*(4), 1043–1052.

Gropper, S. S., & Smith, J. L. (2012). *Advanced nutrition and human metabolism*. Cengage Learning.

Halsted, C. H. (2003). Absorption of water-soluble vitamins. *Current Opinion in Gastroenterology, 19*(2), 113–117.

Herbig, A. L., & Renard, C. M. G. C. (2017). Factors that impact the stability of vitamin C at intermediate temperatures in a food matrix. *Food Chemistry, 220*, 444–451.

Hur, S. J., Lee, S. Y., & Lee, S. J. (2015). Effect of biopolymer encapsulation on the digestibility of lipid and cholesterol oxidation products in beef during *in vitro* human digestion. *Food Chemistry, 166*, 254–260.

Hur, S. J., Lim, B. O., Decker, E. A., & McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry, 125*(1), 1–12.

Kallio, J., Jaakkola, M., Mäki, M., Kilpeläinen, P., & Virtanen, V. (2012). Vitamin C inhibits *staphylococcus aureus* growth and enhances the inhibitory effect of quercetin on growth of *Escherichia coli* in vitro. *Planta Medica, 78*(17), 1824–1830.

Khameneh, B., Bazzaz, B. S. F., Amani, A., Rostami, J., & Vahdati-Mashhadian, N. (2016). Combination of anti-tuberculosis drugs with vitamin C or NAC against different *Staphylococcus aureus* and *Mycobacterium tuberculosis* strains. *Microbial Pathogenesis, 93*, 83–87.

Kjeldgaard, J., Cohn, M. T., Casey, P. G., Hill, C., & Ingmer, H. (2012). Residual antibiotics disrupt meat fermentation and increase risk of infection. *mBio, 3*(5), Article e00190.

Lee, S. Y., Kang, H. J., Kang, J. H., Cho, M. G., Jang, H. W., Kim, B. K., et al. (2021). Differences in the gut microbiota between young and elderly persons in Korea. *Nutrition Research, 87*, 31–40.

Lee, S. Y., Kim, O. Y., Yoon, S. Y., & Hur, S. J. (2018). Changes in resistance to and antimicrobial activity of antibiotics during *in vitro* human digestion. *Journal of Global Antimicrobial Resistance, 15*, 277–282.

Lee, S. Y., Lee, D. Y., & Hur, S. J. (2021). Changes in the stability and antioxidant activities of different molecular weight bioactive peptide extracts obtained from beef during *in vitro* human digestion by gut microbiota. *Food Research International, 141*, 110116.

Lee, S. Y., Lee, D. Y., Kang, H. J., Kang, J. H., Jang, H. W., Kim, B. K., et al. (2021). Analysis of *in vitro* digestion using human gut microbiota in adult and elderly individuals. *Food Chemistry*, 130228.

Lee, S. Y., Lee, S. J., Yim, D. G., & Hur, S. J. (2020). Changes in the content and bioavailability of onion quercetin and grape resveratrol during *in vitro* human digestion. *Foods, 9*(6), 694.

Levi, C. S., & Lesmes, U. (2014). Bi-compartmental elderly or adult dynamic digestion models applied to interrogate protein digestibility. *Food & Function, 5*(10), 2402–2409.

Lopes, G. S., Ferreira, A. T., Oshiro, M. E., Vladimirova, I., Jurkiewicz, N. H., Jurkiewicz, A., et al. (2006). Aging-related changes of intracellular Ca^{2+} stores and contractile response of intestinal smooth muscle. *Experimental Gerontology, 41*(1), 55–62.

Madsen, J. L., & Graff, J. (2004). Effects of ageing on gastrointestinal motor function. *Age and Ageing, 33*(2), 154–159.

Martin, R., Olivares, M., Marin, M., Xaus, J., Fernández, L., & Rodríguez, J. (2005). Characterization of a reuterin-producing *Lactobacillus coryniformis* strain isolated from a goat's milk cheese. *International Journal of Food Microbiology, 104*(3), 267–277.

Nagita, A., Amemoto, K., Yoden, A., Aoki, S., Sakaguchi, M., Ashida, K., et al. (1996). Diurnal variation in intragastric pH in children with and without peptic ulcers. *Pediatric Research, 40*(4), 528–532.

Neale, G. (1990). B12 binding proteins. *Gut, 31*(1), 59.

Obeid, R., Heil, S. G., Verhoeven, M., Van Den Heuvel, E. G., De Groot, L. C., & Eussen, S. J. (2019). Vitamin B12 intake from animal foods, biomarkers, and health aspects. *Frontiers in Nutrition, 6*, 93.

Quadros, E. V. (2010). Advances in the understanding of cobalamin assimilation and metabolism. *British Journal of Haematology, 148*(2), 195–204.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., & International Antioxidant Research Centre, Guy's, King's and St Thomas' School of Biomedical Sciences, Kings College–Guy's Campus, London SE1 9RT, UK. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3. Submitted for publication.

Rémond, D., Shahar, D. R., Gille, D., Pinto, P., Kachal, J., Peyron, M. A., et al. (2015). Understanding the gastrointestinal tract of the elderly to develop dietary solutions that prevent malnutrition. *Oncotarget, 6*(16), 13858.

Rodionov, D. A., Vitreschak, A. G., Mironov, A. A., & Gelfand, M. S. (2003). Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. *Journal of Biological Chemistry, 278*(42), 41148–41159.

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., et al. (2018). Gut microbiota functions: Metabolism of nutrients and other food components. *European Journal of Nutrition, 57*(1), 1–24.

Saidi, B., & Warthesen, J. J. (1983). Influence of pH and light on the kinetics of vitamin B6 degradation. *Journal of Agricultural and Food Chemistry, 31*(4), 876–880.

Said, H. M., & Mohammed, Z. M. (2006). Intestinal absorption of water-soluble vitamins: An update. *Current Opinion in Gastroenterology, 22*(2), 140–146.

Seetharam, B., & Alpers, D. H. (1982). Absorption and transport of cobalamin (vitamin B12). *Annual Review of Nutrition, 2*(1), 343–369.

Smith, C. H., Boland, B., Daureeawoo, Y., Donaldson, E., Small, K., & Tuomainen, J. (2013). Effect of aging on stimulated salivary flow in adults. *Journal of the American Geriatrics Society, 61*(5), 805–808.

Snell, E. E., & Haskell, B. E. (1970). The metabolism of vitamin B6. *Comprehensive Biochemistry, 21*, 47–71. Elsevier.

Swithers, K. S., Petrus, A. K., Secinaro, M. A., Nesbø, C. L., Gogarten, J. P., Noll, K. M., et al. (2012). Vitamin B12 synthesis and salvage pathways were acquired by horizontal gene transfer to the Thermotogales. *Genome Biology and Evolution, 4*(8), 842–851.

Tanner, S. M., Sturm, A. C., Baack, E. C., Liyanarachchi, S., & de la Chapelle, A. (2012). Inherited cobalamin malabsorption. Mutations in three genes reveal functional and ethnic patterns. *Orphanet Journal of Rare Diseases, 7*(1), 1–15.

Uebanso, T., Shimohata, T., Mawatari, K., & Takahashi, A. (2020). Functional roles of B-Vitamins in the gut and gut microbiome. *Molecular Nutrition & Food Research, 64*(18), 2000426.

Vellas, B., Balas, D., Moreau, J., Bouisson, M., Senegas-Balas, F., Guidet, M., et al. (1988). Exocrine pancreatic secretion in the elderly. *International Journal of Pancreatology, 3*(6), 497–502.

Verghese, R., Mathew, S., & David, A. (2017). Antimicrobial activity of Vitamin C demonstrated on uropathogenic *Escherichia coli* and *Klebsiella pneumoniae*. *Journal of Current Research in Scientific Medicine, 3*(2), 88–93.

Zhang, H. M., Wakisaka, N., Maeda, O., & Yamamoto, T. (1997). Vitamin C inhibits the growth of a bacterial risk factor for gastric carcinoma: *Helicobacter pylori*. *Cancer: Interdisciplinary International Journal of the American Cancer Society, 80*(10), 1897–1903.