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Hydrolysis of Chicoric and Caftaric Acids with Esterases and *Lactobacillus johnsonii* in Vitro and in a Gastrointestinal Model

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Supporting Information

ABSTRACT: Chicoric acid (ChA) and caftaric acid (CafA) were identified as bioactive components of chicory and have been ascribed a number of health benefits. This study investigated the hydrolysis of ChA and CafA with enzymes and a probiotic bacterium *Lactobacillus johnsonii* (La1). Esterase from *Aspergillus japonicus* (24 U/mg) hydrolyzed 100% of ChA (5 mM) and CafA (5 mM) after 3 h, at pH 7.0 and 37 °C. Under the same reaction conditions, 100% hydrolysis of ChA and CafA was achieved with a spray-dried preparation of La1. The addition of La1 (100 mg/mL, 3.3 E9 cfu/g) to CafA solution in a gastrointestinal model (GI model) resulted in 65% hydrolysis of CafA. This model simulates the physicochemical conditions of the human gastrointestinal tract. No hydrolysis of CafA was observed after passage through the GI model in the absence of La1. The results of this study support the hypothesis that ChA and CafA are degraded by gut microflora before absorption and metabolization.

KEYWORDS: *chicoric acid, caftaric acid, esterase, gastrointestinal model*

INTRODUCTION

The roots of chicory (*Cichorium intybus* L.) are commercially used for the production of coffee substitutes, mixtures of coffee, and invigorating drinks. Chicory leaves and roots are also widely used as an herbal remedy with a beneficial influence on bile excretion, diuretic action, and gastric juice excretion, as well as stimulation of digestion and metabolism of food ingredients.[1] Chicory root extract has also been found to inhibit the stimulation of PGE_2 synthesis by the pro-inflammatory agent TNF-α, suggesting an anti-inflammatory potential of chicory root.[2] The biological activities of chicory and chicory extracts have been attributed to different compounds such as fructans, polyphenolic acids (e.g., chlorogenic acids, chicoric acid, and caftaric acid), polyphenols, and sesquiterpenes.[3−5] Among these bioactive compounds, chicoric acid (2*R*,3*R*-*O*-dicaffeoyltartaric acid) (ChA) (Figure 1) has been reported to have immunostimulatory properties, antihyaluronidase activity, and a protective effect on the free radical-induced degradation of collagen.[6] Although ChA is commonly thought to occur in *Echinacea purpurea* (purple coneflower) alone, it has been found in numerous sources, including iceberg lettuce, chicory, basil, grape, and grape pomace.[7−10] Caftaric acid (2*R*,3*R*-*O*-caffeoyltartaric acid) (CafA) (Figure 1) is another bioactive phenolic acid identified in chicory. This caffeic acid ester, which is one of the bioactive constituents of *E. purpurea*, was also identified as a major phenolic compound in green- and white-leaf chicory[8] in addition to grapes and wine.[11]

ChA and CafA were reported to be highly susceptible to enzymatic degradation during the preparation of *E. purpurea*.[12] The authors reported that these molecules were oxidized by polyphenol oxidases. They also postulated that ChA and CafA can be hydrolyzed by an esterase from plant origin. This hypothesis has not been confirmed.

The biological properties of hydroxycinnamates (esters of hydroxycinnamic acids, e.g., caffeic, ferulic, *p*-coumaric acids) depend on their absorption and their metabolism. Although the absorption of caffeic acid (CA) in the small intestine has been well characterized in animals and in humans,[13−17] the bioavailability of hydroxycinnamates (e.g., rosmarinic acid, caftaric acid, and chicoric acid) is more controversial.[18] In fact, an animal study showed that *trans*-caftaric acid is rapidly available through the stomach, bypasses the liver, and can reach the brain. It is taken up, metabolized, and excreted as *trans*-fetaric acid (feruloyltartaric acid) by the kidneys.[19] Other in vivo studies suggested that hydroxycinnamates are hydrolyzed in the gut before absorption, by the reported presence of tissue esterases in the upper intestinal mucosa[20,21] and by the microbial esterases in the lower intestinal tract.[22,23] Esterases with the ability to hydrolyze hydroxycinnamates at appreciable rates have been described in humans and rats.[20,21] Bacteria in the gastrointestinal (GI) tract of mammals are also capable of releasing free phenolic acids from bound complexes into the GI tract.[24,25] Indeed, an esterase activity able to hydrolyze chlorogenic acids (esters of caffeic and quinic acids) and release CA has been demonstrated in human colonic microbiota.[22,23] Although there is evidence that hydroxycinnamates are degraded by gut microbiota, only limited information on the microorganisms and enzymes involved in this degradation is available.[22,26] In a previous study, we showed that esterases from *Aspergillus japonicus* and *Lactobacillus johnsonii* (La1) were able to hydrolyze rosmarinic acid.[26] In this study, we investigated the in vitro hydrolysis of ChA and

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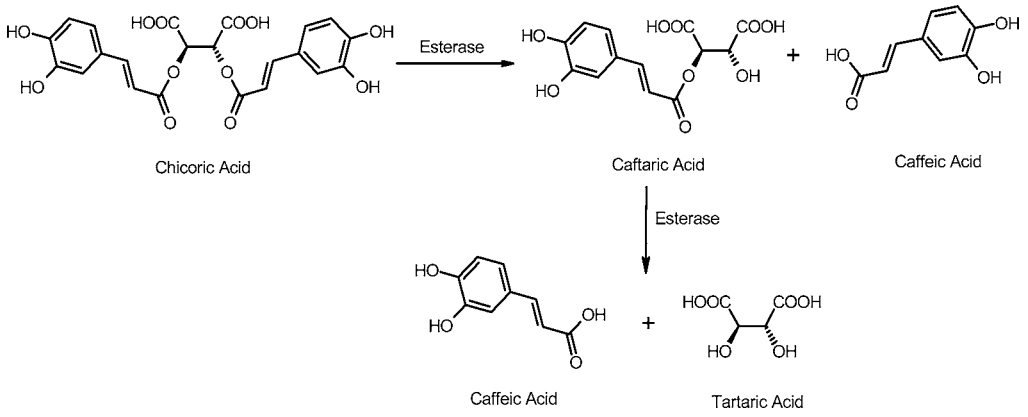

Figure 1. Enzymatic hydrolysis of chicoric acid into caftaric, caffeic, and tartaric acids.

CafA with different esterases and with a probiotic bacterium, *L. johnsonii* NCC 533 (La1). Experiments in a GI model were also conducted to demonstrate that La1 can hydrolyze CafA under physiological conditions. Control experiments, without La1, were performed to see whether this hydrolysis occurs chemically under the conditions of the GI model (e.g., temperature, pH, and bile salts) or is catalyzed by secreted enzymatic activity (e.g., lipase and pancreatic enzymes). The results of this fundamental in vitro study may contribute to a better understanding the fate of ChA and CafA and the role that gut microorganisms can play in this.

MATERIALS AND METHODS

Chemicals. Caffeic, chicoric, caftaric, and tartaric acids were purchased from Sigma-Aldrich (Switzerland).

Enzymes and Microorganisms. Rapidase Maxifruit and Rapidase Press were from DSM (The Netherlands). Flavorase was from Amano Enzymes Inc. (UK). Esterase from *A. japonicus* (24 U/g) was purchased from Kikkoman (Japan). *L. johnsonii* (La1) was from Nestlé Culture Collection (NCC 533).

Hydrolysis of Chicoric and Caftaric Acids with Esterases. Enzymes (10 mg/mL) were dissolved in phosphate buffer (50 mM, pH 7.0) and added to a solution of ChA (10 mM, 4.74 mg/mL) or CafA (10 mM, 3.12 mg/mL) in phosphate buffer (50 mM, pH 7.0). The mixture (100 μL enzyme solution/100 μL substrate solution) was then incubated at 37 °C for 24 h (Eppendorf, Thermomixer). Samples were withdrawn at different reaction times, the enzymatic activity was stopped by heat treatment (5 min, 90 °C), and the mixture was ultrafiltered through microcon filters (microcon centrifugal filter devices, model YM10). The supernatant was then analyzed by high-performance liquid chromatography–diode array detection (HPLC-DAD) and liquid chromatography–mass spectrometry (LC-MS). For all experiments, a reaction control was run in parallel under the same reaction conditions but without enzymes.

Treatment of Chicoric and Caftaric Acids with La1. Ten milligrams of a spray-dried preparation of La1 (3.3 E09 cfu/g) was suspended in 100 μL of phosphate buffer (50 mM, pH 7.0). To this suspension, 100 μL of ChA or CafA solution (10 mM, phosphate buffer 50 mM, pH 7.0) was added. The mixture was then incubated at 37 °C (Eppendorf, Thermomixer), and samples were withdrawn at different reaction times. After centrifugation (3000*g*, 5 min) and filtration (0.22 μm), the samples were analyzed by HPLC-DAD and LC-MS. A reaction control was run in parallel under the same reaction conditions but without bacteria.

Kinetics Modeling. The following model reactions are proposed on the basis of the theory of enzyme kinetics:[27]

$$\text{chicoric} + \text{E} \underset{k_{r1}}{\overset{k_{f1}}{\rightleftarrows}} \text{E–chicoric} \xrightarrow{k_{cat1}} \text{caffeic} + \text{caftaric} + \text{E} \quad (1)$$

$$\text{caftaric} + \text{E} \underset{k_{r2}}{\overset{k_{f2}}{\rightleftarrows}} \text{E–caftaric} \xrightarrow{k_{cat2}} \text{caffeic} + \text{tartaric} + \text{E} \quad (2)$$

$$\text{E–caftaric} + \text{caffeic} \underset{k_{r3}}{\overset{k_{f3}}{\rightleftarrows}} \text{E–caftaric–caffeic} \quad (3)$$

In reaction 1, enzyme (E) binds to ChA to form complex E–chicoric (C1), which is hydrolyzed into CA, CafA, and E. In reaction 2, product CafA competes with ChA by binding to E to form another complex, E–caftaric (C2), which is then decomposed into CA, tartaric, and E. This is a competitive inhibition. In reaction 3, CA product binds to complex C2 to form a ternary dead-end complex E–caftaric–caffeic (C3).

The system of differential equations was integrated numerically. This approach bypasses the assumption of steady states for calculating kinetic parameters V_{max} and K_m. The fitting of experimental data (chicoric, caftaric, and caffeic) to the numerically integrated values of the system of differential equations was done using Berkeley Madonna software v. 8.02. The iteration gives a good convergence and stability. Initial values: chicoric = 384 μM; caftaric = 0; tartaric = 0; caffeic = 0. Initial complex concentrations: E–chicoric = E–caftaric = E–caftaric–caffeic = 0. Fitting parameters: $k_{f1} = 7 \times 10^{-3}$ μM min^{-1}; $k_{r1} = 10^{-2}$ min^{-1}; $k_{f2} = 5.23 \times 10^{-2}$ μM min^{-1}; $k_{r2} = 4.17 \times 10^{-2}$ min^{-1} ; $k_{cat1} = 9.6 \times 10^{-2}$ min^{-1}; $k_{cat2} = 2.1 \times 10^{-2}$ min^{-1}; $k_{f3} = 6.66 \times 10^{-5}$ μM min^{-1}; $k_{r3} = 5.31 \times 10^{-3}$ min^{-1}. Initial enzyme: $E_0 = 140.38$ μM. The system of differential equations used is reported in the Supporting Information. The total CA bound (not quantified by HPLC) can be calculated by the following mathematical equation, where *2 means multiplied by 2 because it contains two molecules of CA.

$$\begin{aligned}&\text{total caffeic acid bound}\\ &= \text{caftaric} + \text{chicoric*2} + \text{E–caftaric} + \text{E–chicoric*2}\\ &\quad + \text{E–caftaric–caffeic*2}\end{aligned}$$

Incubation of Caftaric Acid with La1 in a Gastrointestinal Model. The gastric small-intestinal model (TIM-1) is already described in the literature.[26] The experiments in the model were performed under standard physiological conditions of the gastrointestinal tract. During the experiments, the temperature was kept at 37 °C and salivary, gastric, biliary, and pancreatic secretions were simulated. The digestion process in the model was monitored for 6 h. During the first 3.5 h, the gastric content was gradually delivered into the small intestine "pyloric valve". At the end of the experiment, approximately 80% of the small intestine content was gradually delivered into the "large intestine" via the ileocecal valve. The gastric pH gradually decreased from 6.5 to 2.0 in approximately 5 h by the addition of 1 M HCl; the pH of the small intestinal contents was maintained at 6.5 in the duodenum, at 6.8 in the jejunum, and at 7.2 in the ileum. The products of digestion and water were absorbed from the jejunal and ileal compartments by pumping dialysis liquid through hollow fiber membranes with a molecular weight cutoff of 5000 Da. In a control experiment (run in duplicate), 343 mg of CafA was dissolved

in 300 mL of acetate buffer (20 mM, pH 6.5). After the addition of 10 mL of start residue (5 mL of pepsin (Sigma 600 U/mL) and 5 mL of lipase (Amano 40 U/mL) enzyme solutions), the solution was injected into the gastric compartment of the GI model. During digestion, the total dialysate was collected for 0–2, 2–4, and 4–6 h after passage through the semipermeable hollow-fiber membranes connected to the jejunal and ileal compartments. Total ileal delivery was collected for 0–2, 2–4, and 4–6 h. After the experiment had run for 6 h, the residues from the compartments of the stomach, duodenum, jejunum, and ileum were analyzed to calculate the mass balance of CafA. The samples (2 mL) were passed through 0.45 μm filters and directly analyzed by HPLC-DAD (injection volume = 5 μL). For the experiments with La1, 290 mL of acetate buffer (20 mM, pH 6.5) containing 3 g of a spray-dried preparation of La1 (3.3 E9 cfu/g) (run in duplicate) was put into the gastric compartment after the addition of 10 mL of start residue. In all experiments 10 mL of acetate buffer solution (20 mM, pH 6.5) containing 343 mg of CafA was injected by a syringe into the gastric compartment 15 min after the digestion simulation had been started.

HPLC-DAD and HPLC-MS. HPLC-DAD analysis of ChA, CafA, and hydrolysis products was performed on an Agilent 1100 system equipped with an Atlantis C18 reverse-phase column (4.6 × 100 mm, particle size = 3 μm) and a DAD. The column was equilibrated with water containing 0.1% formic acid. After injection, a linear gradient to a final solvent composition of 55% water and 45% acetonitrile (containing 0.1% formic acid) was run within 12 min at a flow rate of 1 mL/min. ChA, CafA, and CA were monitored by UV at 320 nm and quantified using standard calibration curves.[26] HPLC-MS analysis was performed on a Waters Alliance 2695 HPLC system equipped with a Macherey-Nagel Nucleosil 120-3 C18 column (3 μm particle size, 120 Å pore size, and 4 mm × 250 mm). The eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Used was the following gradient: from 0 to 15 min, linear gradient to 50% A; from 15 to 40 min, isocratic 50% A. The flow rate was kept constant at 400 μL/min. The column was equilibrated for 6 min with 100% A. HPLC-DAD was used to quantify ChA, CafA, and CA, whereas HPLC-MS was used to detect any other degradation product (e.g., oxidation).

RESULTS

Hydrolysis of Chicoric Acid with Esterases. The hydrolysis of ChA was performed with different esterases. As shown in Figure 1, this hydrolysis resulted in the generation of CA and CafA, which was then transformed into CA and tartaric acid. The kinetics of the generation of CafA and CA from ChA was studied at pH 7.0 and 37 °C using an esterase from *A. japonicus* as biocatalyst (Figure 2). Under these reaction conditions, ChA was completely hydrolyzed after 3 h of incubation (reaction yield = 40%). The complete hydrolysis of generated CafA occurred only after >8 h of reaction time (Table 1). The pH dropped from 7.0 to 6.5. Among tested esterases, Rapidase and *A. japonicus* esterase were able to perform complete hydrolysis of ChA in <8 h of reaction time (Table 1). The hydrolysis rate of ChA was very low with Flavorase, whereas almost no hydrolysis was observed with Rapidase Press even after 24 h of reaction time. No hydrolysis of ChA was observed in the reaction controls.

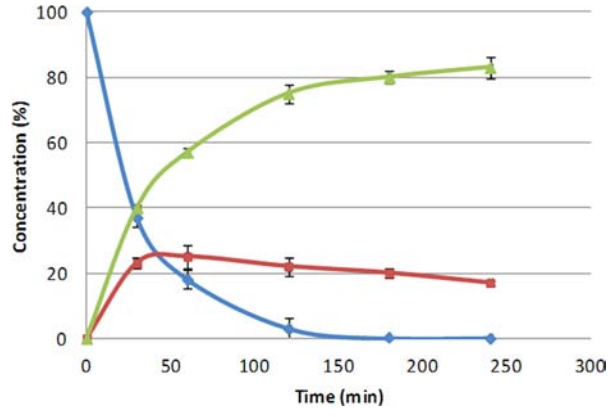

Figure 2. Hydrolysis of ChA (blue diamonds) (5 mM) into CafA (red squares) and CA (green triangles) by esterase from *A. japonicus* at 37 °C and pH 7.0. Values are the mean of two independent experiments.

Table 1. Hydrolysis (Percent) of Chicoric and Caftaric Acids by Esterases and La1[a]

	chicoric acid			caftaric acid		
	4 h	8 h	24 h	4 h	8 h	24 h
A. japonicus esterase	100	100	100	100	100	100
Rapidase	95	100	100	60	82	98
Flavorase	46	65	92	12	18	50
Rapidase Press	8.0	10	12	0.0	2.0	3.0
L. johnsonii	95	100	100	84	97	100

[a]Values are the mean of three independent experiments. For all values, SD ≤ 2.0.

Hydrolysis of Chicoric Acid with *L. johnsonii* NCC 533 (La1). As the hydrolysis of ChA with probiotic bacteria has never been reported, we investigated this hydrolysis by La1. As shown in Figure 3, >95% of ChA was hydrolyzed after 4 h of reaction time. The hydrolysis of ChA resulted in the generation of CA and CafA, which was then transformed into CA and tartaric acid (total reaction yield = 37%).

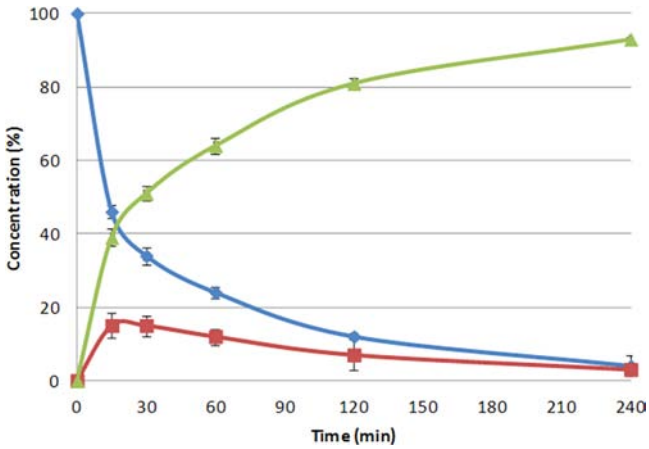

Figure 3. Hydrolysis of ChA (blue diamonds) (5 mM) into CafA (red squares) and CA (green triangles) by La1 (100 mg/mL, 3.3×10^9 cfu/g) at 37 °C and pH 7.0. Values are the mean of two independent experiments.

Kinetics Modeling. Theoretically, the complete hydrolysis of one molecule of ChA should result in the generation of two molecules of CA and one molecule of tartaric acid from the mass balance point of view (Figure 1). This means that from an initial concentration of 384 μM ChA, the final concentration of CA should be 768 μM if complete hydrolysis is reached. From the experimental data (Figure 4, marker points), it can be seen that the amount of CA was only about 450 μM and no degradation products (e.g., oxidation products) were identified in the reaction media by LC-MS.

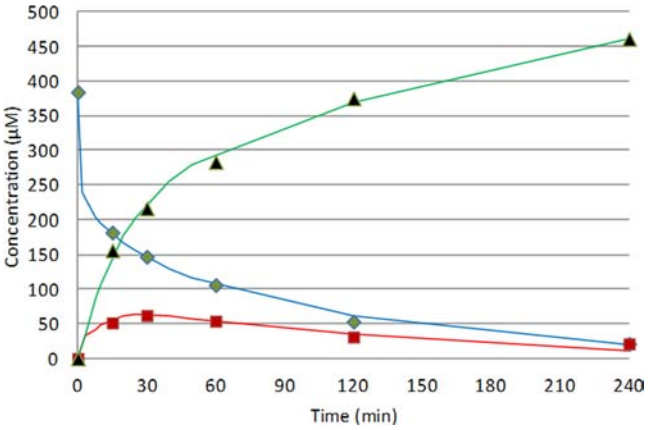

Figure 4. Hydrolysis of ChA (384 μM) with esterase from *A. japonicus* at 37 °C and pH 7.0. Comparison of measured concentrations of ChA (green diamonds), CafA (red squares), and CA (black triangles) with calculated concentrations of ChA (blue curve), CafA (red curve), and CA (green curve). The results presented in this figure are from different experiments from those presented in Figure 2.

To explain this behavior, intermediates produced by the binding of various molecules to the enzyme have to be taken into account. A series of various types of inhibition is described in the proposed reaction model described under Materials and Methods. By calculations, the complex C1 (E−chicoric) shows a maximum of 76 μM at 12 min and decreases slowly to 7 μM at 100 min (data not shown), whereas the complex C2 (E−caftaric) increases to a maximum of 87 μM at 30 min and very slowly decreases to 18 μM (data not shown). Caffeic acid was then assumed to associate with complex C2 to form a ternary dead-end complex C3 (E−caftaric−caffeic). The concentration of complex C3 rises to a plateau of about 115 μM (data not shown); in this case CA uncompetitively inhibits the hydrolysis of CafA. All forms of total bound CA drop from an initial concentration of 768 to 268 μM. This may explain the reduced concentration of CA measured by HPLC in the reaction media. The data shown in Figure 4 support this hypothesis as the measured (marker points) and calculated (lines) concentrations of ChA, CafA, and CA were very well correlated. On the basis of these results we propose that CA uncompetitively inhibits the hydrolysis of CafA by forming the complex E−caftaric−caffeic.

Hydrolysis of Caftaric Acid with Esterases and La1. Among tested esterases, only the esterase from *A. japonicus* was able to perform complete hydrolysis of CafA after 4 h of reaction time at pH 7.0 and 37 °C (Table 1). Under the same conditions, the hydrolysis rate of CafA was very low with Flavorase, and no hydrolysis was observed with Rapidase Press. More than 95% of CafA was hydrolyzed after 8 h of reaction time when a spray-dried preparation of La1 (3.3 E9 cfu/g) was used at a concentration of 100 mg/mL, at pH 7.0 and 37 °C.

Hydrolysis of Caftaric Acid with La1 in a GI Model. A spray-dried preparation of La1 (3.3×10^9 cfu/g) was used to hydrolyze CafA in the GI model. CafA was injected into the gastric compartment 15 min after the digestion simulation had been started. Fractions were collected at different time courses. After 6 h, 65% of CafA was hydrolyzed in the efflux samples (Table 2). The hydrolysis of CafA by La1 esterase started in the stomach and continued in the ileal and jejunal parts of the GI model (Table 2). In a control experiment, CafA was applied to digestion in the GI model without the addition of La1 to see whether hydrolysis occurs chemically under the conditions of the GI model (37 °C, low pH, and bile salts) or is catalyzed by secreted enzymatic activity (lipase and pancreatic enzymes). During the 6 h experiment, no hydrolysis of CafA was observed by HPLC analyses.

Table 2. Hydrolysis (Percent) of Caftaric Acid by La1 in a GI Model[a]

	1 h	2 h	4 h	6 h
jejunal dialysate		17	35	42
ileal dialysate		18	38	55
stomach	15			
efflux				65

[a]Values are the mean of two independent experiments. For all values, SD ≤ 2.0.

DISCUSSION

Hydroxycinnamic acids (e.g., caffeic, ferulic, *p*-coumaric acids) constitute a major class of polyphenols widely distributed in fruits, vegetables, and beverages (e.g., wine, coffee, and chicory). CA is one of the most abundant hydroxycinnamic acids in the human diet. It can be found free or esterified with either quinic acid (e.g., chlorogenic acids) or tartaric acid (e.g., ChA, CafA). The absorption and metabolism of chlorogenic acids have been studied in animals, humans, and human tissues.[28] Studies have shown that after consumption of chlorogenic acid-containing fruits by human subjects, CA metabolites, but not chlorogenic acid, were found in urine. In rats fed chlorogenic acids, CA conjugates, but not chlorogenic acid, were recovered in the plasma. CA is the main metabolite detected in human urine after the ingestion of pure chlorogenic acids. These results show that after release from chlorogenic acid by gut microbial esterases, CA is not further metabolized by the microflora, but can be absorbed into the human body and conjugated, presumably by UDP-glucuronyl transferases.[22] Unlike chlorogenic acids, only limited information on the bioavailability and metabolism of ChA and CafA is available. A human study has shown that following intake of a single serving of white wine containing 162 μmol of tartaric esters (e.g., ChA and CafA), free, glucuronidated, and sulfated caffeic, ferulic, and *p*-coumaric acids were present in the plasma, but no intact esters were detected.[11] These plasma and urinary profiles of tartaric ester metabolites were also reported recently in a human study following the intake of grape juice.[29] Therefore, gut bacterial enzymes able to hydrolyze the ester bond in hydroxycinnamates could play a key role in the potential health benefits of this class of compounds. The enzymatic hydrolysis of the ester bond in hydroxycinnamates results in the generation of CA, which has antioxidant properties, illustrated by its ability to scavenge various free radicals when tested in vitro.[30] In vivo when ingested with the diet, CA increases the plasma antioxidant capacity, the concentration of endogenous antioxidants such as vitamin E, and the ex vivo resistance of lipoproteins to oxidation.[18,31,32] In vivo and in vitro studies have demonstrated that CA binds to serum albumins more extensively than ferulic acid, *m*-coumaric acid, *p*-coumaric acid, and 5-CQA.[28]

In the present investigation, ChA and CafA were enzymatically hydrolyzed into CA and tartaric acid. Among tested enzymes, esterase from *A. japonicus* was the most efficient, and after <4 h of reaction time, complete hydrolysis of ChA and CafA was achieved under the reaction conditions used in this

study. This enzymatic hydrolysis of ChA and CafA could be an interesting approach for applications in food and beverage products. Esterases able to hydrolyze hydroxycinnamates have been found in bacteria from human and animal intestinal microbiota.[22] *L. johnsonii* NCC533 (La1) used in this study is a probiotic bacterium of human origin, is safe for consumption, adheres to human intestinal cell lines, survives in the human gastrointestinal tract and colonizes temporarily in the intestine, produces antimicrobial substances, antagonizes pathogenic bacteria, and has beneficial effects on human health.[33] More than 95% of ChA and CafA was hydrolyzed during the incubation with a spray-dried preparation of La1 after 8 h of reaction time. Thus, some strains present in gut microbiota may play a crucial role in the bioavailability of poorly absorbed polyphenols and may contribute to their health effects. By studying the kinetics of the hydrolysis of ChA with *A. japonicus* and La1 esterases, we observed that the concentration of CA generated was much lower than the expected one. To explain this behavior and on the basis of the theoretical calculations, we propose that CA uncompetitively inhibits the hydrolysis of CafA. Experiments in the GI model were conducted to see whether the hydrolysis of CafA occurs chemically under the conditions of the GI model (e.g., temperature, pH, and bile salts), catalyzed by secreted enzymatic activity (e.g., lipase, pancreatic enzymes) or by selected enzymes and microorganisms. No hydrolysis of CafA was observed under the physiological conditions mimicked in the GI model. The hydrolysis of CafA was not catalyzed by secreted enzymatic activity. However, substantial hydrolysis of CafA was observed when La1 was added in the GI model. These results confirm the hypothesis that CafA and probably other hydroxycinnamates are degraded by gut microflora before their absorption and are then metabolized in various tissues such as the intestine, liver, and kidney. The results obtained in this study support the hypothesis that ChA and CafA could be degraded by human gut microflora before absorption and metabolization. The combination of La1 and ChA or CafA might result in increased in vivo bioavailability of CA. This hypothesis has to be further confirmed by clinical studies.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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