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ARTICLE

Hydrolysis of Rosmarinic Acid from Rosemary Extract with Esterases and *Lactobacillus johnsonii* in Vitro and in a Gastrointestinal Model

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Rosmarinic acid (RA) was identified as one of the main components of rosemary extracts and has been ascribed to a number of health benefits. Several studies suggested that after ingestion, RA is metabolized by gut microflora into caffeic acid and derivatives. However, only limited information on the microorganisms and enzymes involved in this biotransformation is available. In this study, we investigated the hydrolysis of RA from rosemary extract with enzymes and a probiotic bacterium *Lactobacillus johnsonii* NCC 533. Chlorogenate esterase from *Aspergillus japonicus* (0.02 U/mg) hydrolyzed 90% of RA (5 mg/mL) after 2 h at pH 7.0 and 40 °C. Complete hydrolysis of RA (5 mg/mL) was achieved with a preparation of *L. johnsonii* (25 mg/mL, 3.3 E9 cfu/g) after 2 h of incubation at pH 7.0 and 37 °C. No hydrolysis of RA was observed after the passage of rosemary extract through the gastrointestinal tract model (GI model). Thus, RA is hydrolyzed neither chemically under the conditions of the GI model (temperature, pH, and bile salts) nor by secreted enzymatic activity (lipase and pancreatic enzymes). The addition of *L. johnsonii* cells to rosemary extract in the GI model resulted in substantial hydrolysis of RA (up to 99%).

KEYWORDS: Rosmarinic acid; *Lactobacillus johnsonii*; probiotic; esterase; gastrointestinal model

INTRODUCTION

Rosemary was widely studied and used in many applications as an herbal remedy with antioxidant, anti-inflammatory, antimicrobial, anticarcinogenic, and hepatoprotective properties (*1*−*4*). These biological activities have been attributed to different compounds such as monoterpenes (e.g., etheric oils), diterpenes (e.g., carnosic acid, carnosol, and rosmanol), phenolic acids [e.g., rosmarinic acid (RA)], flavonols, and flavones (*5*, *6*). Among these bioactive compounds, RA has been reported to have in vitro antioxidative activity such as the ability to scavenge superoxide and hydroxyl radicals (*7*) and inhibit oxidation of low-density lipoprotein (*8*). In addition, RA has been shown to have several other biological activities including a potent anti-inflammatory effect resulting from decreased arachidonate formation (*9*), inhibition of hemolysis (*10*), and suppression of hyaluronidase and β-hexoaminidase (*11*). Most recent studies showed other biological effects of RA such as photoprotection against UV (*12*), protection of human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis (*13*), antiactivator protein-1-dependent activation of COX-2 expression in nonmalignant cell lines (*14*), effect on atopic dermatitis (*15*), and induction of apoptosis of activated T cells (*16*). In vivo studies were performed to understand the metabolism and bioavailability of RA (*17*). In rats, orally administered RA was present as intact, degraded, and/or conjugated forms such as *m*-hydroxyphenylpropionic acid, *m*-coumaric acid, and sulfated forms of caffeic acid (CA) and ferulic acid (FA) that are subsequently excreted in the urine (*18*). In healthy subjects, the plasma concentration and urinary excretion of RA were studied after ingestion of *Perilla frutescens* (*19*). The authors suggested that hydrolysis is one of the pathways of RA metabolism. The resulting CA is then absorbed, conjugated, and methylated in tissues such as the digestive tract and liver, resulting in a variety of metabolites. The hypothesis that phenolic acids like RA are transformed by enzymes or gut microflora before their absorption was also proposed by other animal or human studies (*20*−*22*). Although there is evidence that phenolic acids (e.g., RA) are degraded by gut microflora before their absorption, only limited information on the microorganisms and enzymes involved in this degradation is available.

In this study, we investigated the in vitro hydrolysis of RA with different esterases and with a probiotic bacterium *Lactobacillus johnsonii* NCC 533 (La1). Experiments in the gastrointestinal (GI) tract model (TIM-1) were also conducted to see whether this hydrolysis occurs chemically under the conditions of the GI model (e.g., temperature, pH, and bile salts), catalyzed by secreted enzymatic activity (e.g., lipase and pancreatic enzymes), or by selected enzymes and microorganisms. The results of this fundamental in vitro study may contribute to better understanding the fate of RA and the role that microorganisms can play in this.

MATERIALS AND METHODS

Chemicals. RA, CA, and FA were purchased from Sigma-Aldrich (Switzerland). Rosemary extract PE 20 was from Naturex (France).

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Enzymes and Microorganisms. Esterase from *Saccharomyces cerevisiae* (2.2 U/g), hog liver esterase immobilized on Eupergit C (205 U/g), and porcine liver esterase PLE (130 U/g) were from Fluka (Buchs, Switzerland). Chlorogenate esterase from *Aspergillus japonicus* (24 U/g) was purchased from Kikkoman (Japan). *L. johnsonii* (La1) was from Nestlé Culture Collection (NCC 533).

Culture Conditions. *L. johnsonii* La1 (NCC 533) was cultivated in Man Rogosa Sharpe (MRS) broth (Difco, Basel, Switzerland) at 37 °C under anaerobic conditions and without agitation. Cells were harvested after 16 h of incubation by centrifugation at 5000*g* for 10 min at 4 °C.

Hydrolysis of RA with Enzymes. RA (5 mg/mL) and enzyme (0.01, 0.02, or 1 U/mg RA) were dissolved in 2 mL of phosphate buffer (10 mM, pH 6.5) and incubated at 40 °C (Eppendorf, thermomixer) for 24 h. Samples were withdrawn at different reaction times. The influence of ratio [E]/[S], temperature, and pH on the reaction rate and yield were studied. Soluble enzymes were ultrafiltered through microcon filters (microcon centrifugal filter devices model YM-10, Millipore), while immobilized enzymes were filtered (0.22 μm). In both cases, the supernatants were analyzed by liquid chromatography [high-performance liquid chromatography−diode array detection (HPLC-DAD)]. For all of the experiments, a reaction control was run in parallel under the same conditions but without enzyme or bacteria.

Treatment of RA with La1. After the growth of bacteria and centrifugation (5000*g*, 10 min), the pellet was suspended in phosphate buffer (50 mM, pH 8.0) at a concentration of 0.61 g/mL. RA was then added (5 mg/mL), and the mixture was incubated at 37 °C (Eppendorf, thermomixer). Samples were withdrawn at different reaction times, centrifuged (14000*g*, 10 min), filtered (0.22 μm), and analyzed by HPLC-DAD and liquid chromatography−mass spectrometry (LC-MS). The influence of pH, temperature, and cell concentration on reaction rate and yield was studied.

Treatment of RA with La1 Crude Extract. After culture of La1 and centrifugation (5000*g*, 10 min), the pellets were suspended in phosphate buffer (50 mM, pH 8.0) at a concentration of 0.61 g/mL. The cells were then lysed using the glass beads method. Six hundred microliters of cell preparation (0.61 g/mL) was put in special tubes (Sarstedt Mikro-Schraubgefässe 72.693.005), and 600 μL of glass beads was added at 0 °C. The tubes were then put into a mini-beadbeater for 1 min, cooled in ice, and put into in the mini-beadbeater for another 1 min. This crude cell extract (0.5 mL) was then incubated with 2.5 mg of RA at 37 °C (Eppendorf, thermomixer). Samples were withdrawn at different reaction times, centrifuged (14000*g*, 10 min), filtered (0.22 μm), and analyzed by HPLC. The influence of pH, concentration of La1, and temperature on reaction rate and yield was studied.

Incubation of Rosemary Extract with La1. Rosemary extract Naturex PE 20 (25 mg, 20% RA) was dissolved in 1 mL of phosphate buffer (50 mM, pH 7.0). To 250 μL of this solution, 5 mg of La1 cells (3.3 E9 cfu/g) dissolved in 250 μL of phosphate buffer (50 mM, pH 7.0) was added, and the mixture was incubated at 37 °C (Eppendorf, thermomixer). Samples were withdrawn at different reaction times, centrifuged (14000*g*, 10 min), diluted 20 times, filtered (0.22 μm), and analyzed by HPLC-DAD and LC-MS. A reaction control was run in parallel under the same conditions but without bacteria.

Incubation of Rosemary Extract with La1 in GI Model (TIM-1). The TNO gastric small intestinal model (TIM-1) has already been described in the literature (*23*). This GI model comprises four connected compartments that represent the stomach, duodenum, jejunum, and ileum, respectively. Each compartment consists of a glass outer wall with a flexible inner wall. The flexible wall is surrounded by water at 37 °C to squeeze the walls, which ensures mixing of the food with the secreted enzymes by peristaltic movements in the GI tract. The experiments in the model were performed under standard physiological conditions of the GI 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 secretion of 1 M HCl; the pH of the small intestinal contents was maintained at 6.5 in the duodenum, 6.8 in the jejunum, and 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 mass cutoff of 5000 Da. In a control experiment (run in duplicate), 3 g of rosemary extract (Naturex PE 20) 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 TIM-1. 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 running the experiment for 6 h, the residues from the compartments of the stomach, duodenum, jejunum, and ileum were analyzed to calculate the mass balance of RA. The samples (2 mL) were passed through 0.45 μm filters for cleanup 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 a total of 1.8 E12 cfu/g (run in duplicate) and 2.3 E11 cfu/g of fresh cells was put into the gastric compartment after the addition of 10 mL of start residue, respectively. In all experiments, 10 mL of acetate buffer solution (20 mM, pH 6.5) containing 1.5 g of rosemary extract (Naturex PE 20) was injected by a syringe into the gastric compartment 15 min after starting the digestion simulation. During digestion, 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. Aliquots (1 mL) were taken from the gastric compartment directly after the addition of rosemary extract and at a time point of 1 h. After 6 h, the residues from the compartments of the stomach, duodenum, jejunum, and ileum were analyzed to calculate the mass balance of RA. The samples (2 mL) were passed through 0.45 μm filters for cleanup and directly analyzed by HPLC-DAD (injection volume, 5 μL).

HPLC-DAD and HPLC-MS. PLC-DAD analysis of RA and hydrolysis products was performed on a Agilent 1100 system equipped with a Atlantis C18 reverse-phase column (4.6 mm × 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. RA and CA were monitored by UV at 320 nm, whereas 3,4-dihydroxyphenyllactic acid (DHPL) was monitored at 280 nm. RA and CA were quantified using standard calibration curves as follows: RA and CA (commercial standards) were dissolved in a solution containing 25% acetonitrile and 75% water solution containing citric acid (75 mM) and ammonium acetate (25 mM). RA and CA were injected at 1, 2.5, 5, 10, and 20 μM for the calibration curve. As DHPL was not commercially available, its quantification was based on the calibration curve of CA. 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, 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. A Micromass Q-TOF II hybrid mass spectrometer equipped with an atmospheric pressure chemical ionization source was used for detection. Experimental parameters were as follows: nebulizer gas (nitrogen) maximum (approximately 900 L/h); auxiliary gas, 250 L/h; cone gas, 75 L/h; source block temperature, 150 °C; nebulizer temperature, 400 °C; TOF potential, 9.1 kV; MCP potential, 2200 V. A 3.5 kV corona voltage and 30 V cone voltage were used in negative ion mode. The mass range was m/z 50−1000 Th, the scan time was 1 s, and the resolution was 8000. Transmitter quadrupole parameters were as follows: $mass_1$, 50 AMU; $time_1$, 10%; $ramp_1$, 5%; $mass_2$, 170 AMU; $time_2$, 85%; $ramp_2$, 0%; and $mass_3$, 300 AMU. Argon was used as the focusing/collision gas at a pressure of 12 psi. The focusing/collision energy was 10 eV. RA and CA were identified by comparison of their retention times and mass spectra with reference compounds. DHPL was identified by comparison of its mass spectrum with literature data.

RESULTS

Hydrolysis of RA with Esterases. Among tested esterases, only chlorogenate esterase was able to hydrolyze RA. The hydrolysis resulted in the generation of CA and DHPL (**Figure 1**). The influence of pH and temperature on the reaction rate and yield was studied. **Figure 2** shows the generation of CA from RA at the pH values of 5.0, 6.0, 7.0, and 8.0. RA was completely hydrolyzed after 2 h at pH 8.0 (reaction yield 96%), while at pH 5.0 and 6.0 the reaction rate was very low. No hydrolysis of RA was observed in the reaction controls. The maximum esterase activity was observed between 40 and 50 °C, when the reaction was performed at pH 7.0 using enzyme at 0.02 U/mg (data not shown).

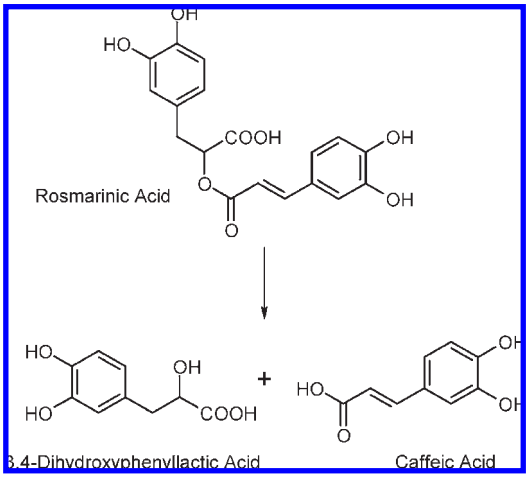

Figure 1. Hydrolysis of RA into CA and DHPL.

Treatment of RA with *L. johnsonii* NCC 533. As the hydrolysis of RA with probiotic bacteria has never been reported, we investigated this hydrolysis by La1. As shown in **Figure 3**, more than 80% of RA was hydrolyzed after 6 h of reaction time when La1 (3.3 E9 cfu/g) was used at a concentration of 152 mg/mL, at pH 8.0 and 37 °C. The reaction yield was 92%. This is the first time that cinnamoyl esterase-like activity has been experimentally demonstrated in La1. The hydrolysis of RA resulted in the formation of CA and DHPL. The concentration of CA was proportional to the concentration of hydrolyzed RA. All molecules RA, CA, and DHPL were stable under the reaction conditions used in this experiment. The hydrolysis of RA with La1 was achieved at room temperature; however, the reaction rate was significantly higher between 40 and 50 °C (data not shown).

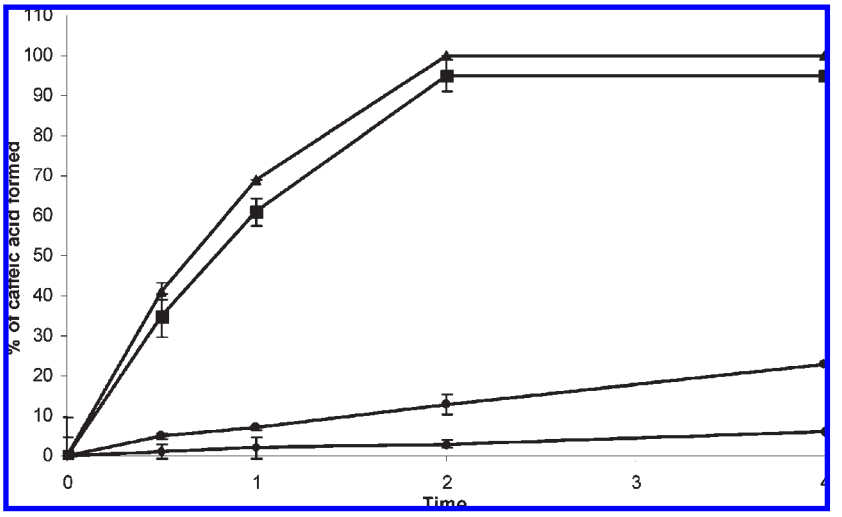

Figure 2. Influence of pH on the formation of CA from RA by chlorogenate esterase (0.02 U/mg) at 37 °C and at the following pH values: 5.0 (●), 6.0 (■), 7.0 (▲), and 8.0 (♦). Values are means of two independent experiments.

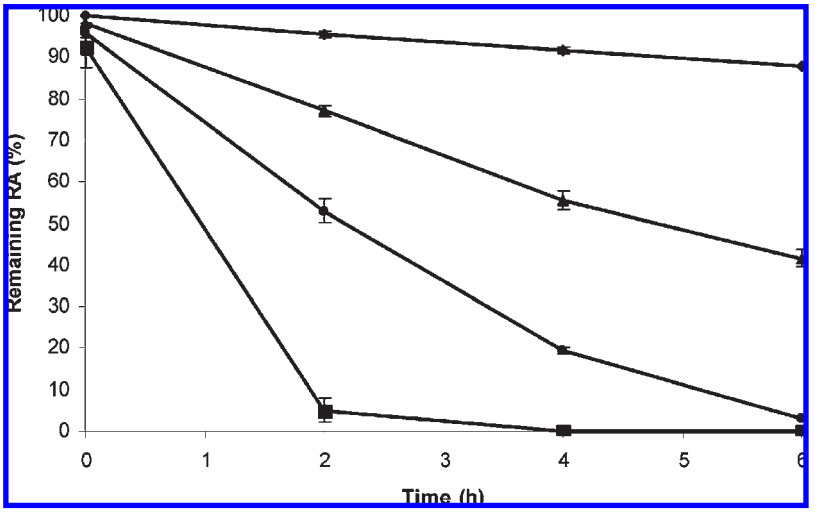

Figure 3. Hydrolysis of RA (2.5 mg/mL) at 37 °C and pH 7.0 with different concentrations of La1: 1 (●), 5 (▲), 10 (♦), and 25 mg/mL (■). Values are means of two independent experiments.

The influence of pH on the hydrolysis of RA with La1 is shown in **Figure 4**. La1 cinnamoyl esterase-like activity showed maximum activity close to pH 7.0 and was most strongly inhibited by low pH.

Treatment of RA with *L. johnsonii* Crude Extract. Incubation of RA with La1 crude extract resulted in a strong increase of the reaction rate as compared to nonlysed cells. In fact, after only 5 min (RA, 5 mg/mL; La1, 152 mg/mL), RA was completely hydrolyzed (**Figure 5**). This result suggests that the enzyme involved in the hydrolysis of RA is presumably intracellular.

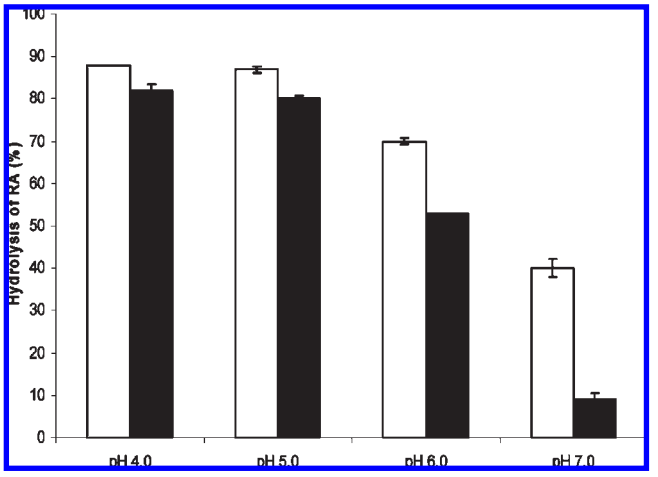

Figure 4. Influence of pH on the hydrolysis of RA (5 mg/mL) by La1 (10 mg/mL) at 37 °C after 2 (□) and 4 h (■).Values are means of two independent experiments.

Incubation of Rosemary Extract with *L. johnsonii*. Rosemary extract (Naturex PE) was incubated with a preparation of La1 (3.3 E9 cfu/g) at 37 °C and pH 7.0. After 4 h, RA was completely hydrolyzed into CA and DHPL (92% yield). This is illustrated by the results of HPLC analysis (**Figure 6**). The chromatograms also reveal that this reaction is the most dominant in the extract, and other minor constituents are relatively well-preserved after the reaction.

Treatment of Rosemary Extract with La1 in a GI Model (TIM-1). In a first experiment (**Figure 7**), La1 (2.3 E12 cfu/g) was used to hydrolyze RA in the TIM-1 model. To avoid hydrolysis of RA prior to introduction of the meal into the gastric compartment, the rosemary extract solution was added only 15 min after starting the digestive process. In the fractions collected from 0 to 2 h, 82% of RA for the jejunal dialysate and 95% for the ileal dialysate and ileal efflux were hydrolyzed. In the fractions collected from 2 to 4 h, only 4% RA remained intact in the jejunal dialysate, while RA was completely hydrolyzed in the ileal samples. In the fractions collected between 4 and 6 h, RA could not be detected in any of the samples (**Figure 7**). As the hydrolysis occurred rapidly, the concentration of La1 was reduced in a second experiment (1.8 E11 cfu/g) (**Figure 8**). In the fractions

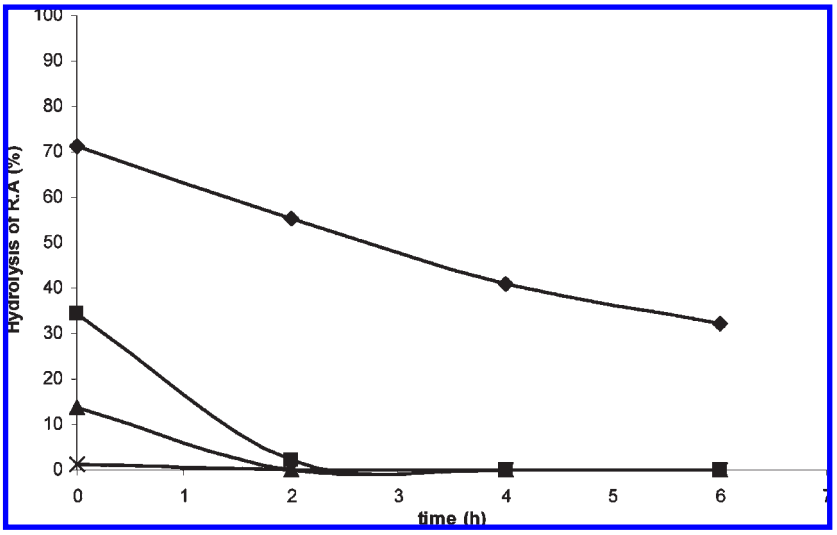

Figure 5. Hydrolysis of RA (5 mg/mL) at 37 °C and pH 8.0 with different concentrations of La1 crude extract: 30.5 (♦), 61 (■), 91 (▲), and 152 mg/mL (×).

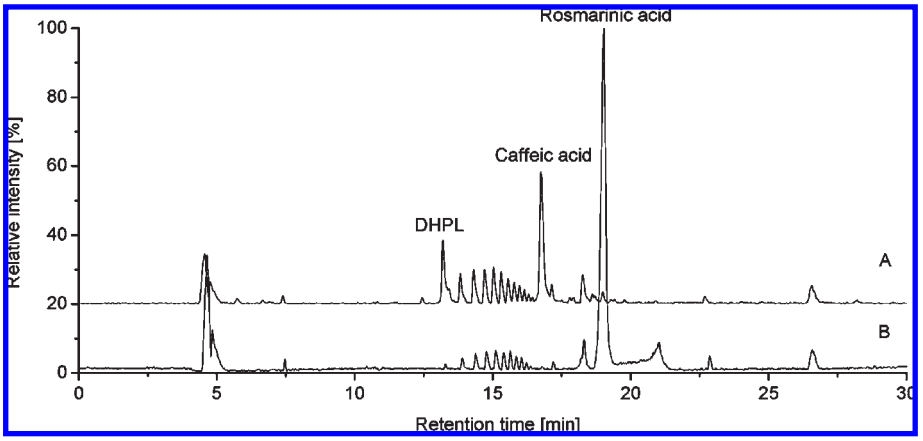

Figure 6. HPLC chromatogram of rosemary extract before (**A**) and after (**B**) treatment with La1 at 37 °C and pH 7.0 for 4 h.

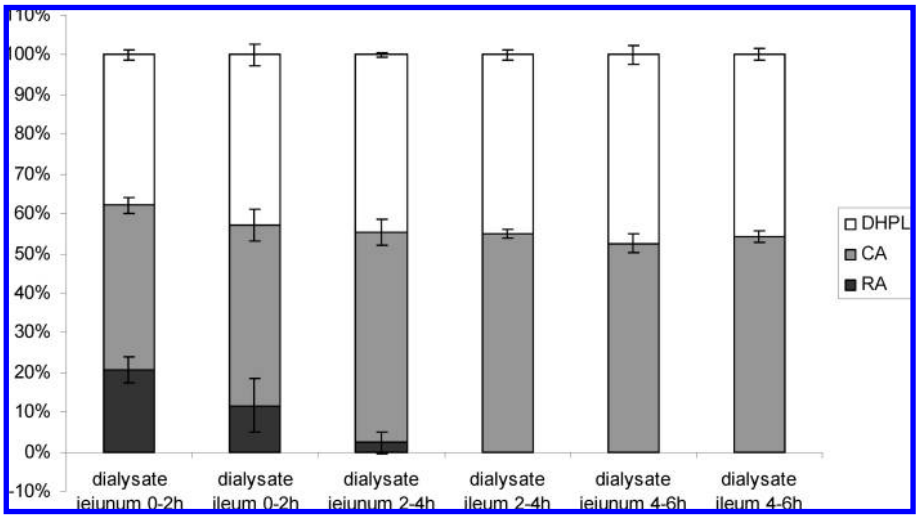

Figure 7. Ratio of RA, CA, and DHPL for the jejunal and ileal dialysates at 0−2, 2−4, and 4−6 h as determined by HPLC-DAD analysis. Digestion experiments for 6 h were performed with La1 (2.3 E12 cfu/g) and rosemary extract (3 g) in the TIM-1 model. Values are means of two independent experiments.

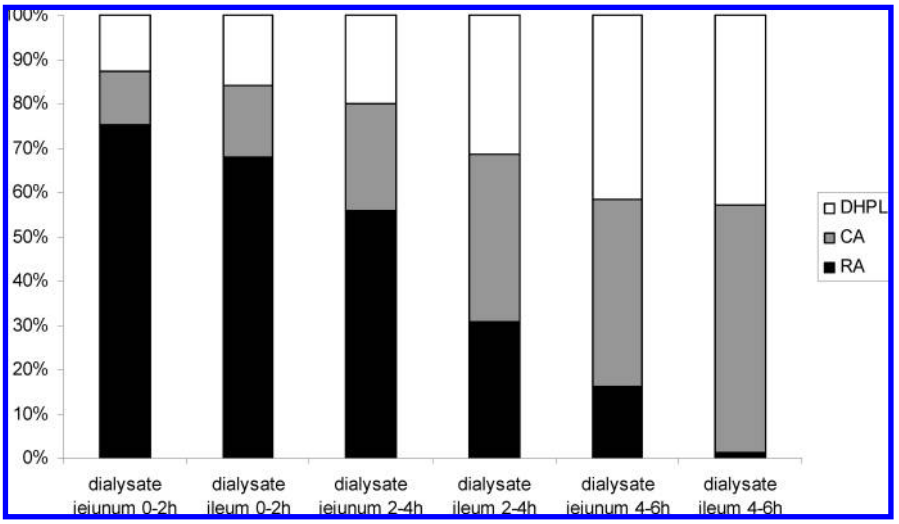

Figure 8. Ratio of RA, CA, and DHPL for the jejunal and ileal dialysates at 0−2, 2−4, and 4−6 h as determined by HPLC-DAD analysis. Digestion experiments for 6 h were performed with La1 (1.8 E11 cfu/g) and rosemary extract (3 g) in the TIM-1 model.

collected from 0 to 2 h, 25% of RA for the jejunal dialysate and 31% for the ileal dialysate and ileal efflux were hydrolyzed; in those collected from 2 to 4 h, 44% of RA for the jejunal dialysate and 69% for the ileal dialysate and efflux were hydrolyzed. In the fractions collected from 4 to 6 h, 84% of RA for the jejunal dialysate and 99% for the ileal dialysate and efflux were hydrolyzed. For both experiments, no significant hydrolysis of RA was observed in samples taken from the stomach compartment (data not shown). In a control experiment, rosemary extract was applied to digestion in the TIM-1 without 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 RA was observed by HPLC analyses. Small quantities (0.6%) of CA were already present in the rosemary extract and did not increase during the experiment, whereas DHPL could not be detected in any of the samples analyzed.

DISCUSSION

The leaves of the plant *Rosmarinus officinalis* L. are well-known as spices and flavoring agents. They have also been reported as herbal remedies with antioxidant, anti-inflammatory, anticarcinogenic, and hepatoprotective properties. These biological effects have been related to diterpenes and polyphenols such as RA, which has been reported to have in vitro antioxidative and anti-inflammatory activities.

Studies in rats have shown that orally administered RA was present as intact and degraded and/or conjugated forms. In a human study (*19*), the authors proposed a hypothetical metabolism for RA. They suggested that the metabolism of RA involves microbial esterase in the digestive tract, hydrolyzing the ester linkage in RA. The resulting CA and coumaric acid derived from RA are then absorbed, conjugated, and methylated in tissues such as the digestive tract and liver, resulting in a variety of metabolites such as glucuronidated or sulfated conjugates. In other studies (*24*, *25*), it has been shown that gut bacterial esterases are able to hydrolyze the ester bond in hydroxycinnamates. These enzymes could play an important role in the uptake and the potential health benefits of this class of molecules. Although there is evidence that polyphenols such as RA are degraded by gut microflora before their absorption and metabolism, only limited information on the microorganisms and enzymes involved in these biotransformations is available.

Recently (*26*), food and human intestinal bacterial strains were screened for cinnamoyl esterase activity. The enzymatic hydrolysis of RA results in the generation of CA and DHPL. CA has antioxidant properties, illustrated by its ability to scavenge various free radicals when tested in vitro (*27*, *28*). 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 (*29*−*31*).

DHPL is one of the most widely used traditional Chinese medicines for the treatment of various cardiovascular diseases and has been reported to have potential protective effects from oxidative injury (*32*, *33*). DHPL has also been used for its radical scavenging and antioxidant activities as well as for the treatment of cerebrovascular diseases (*34*). In the present investigation, RA was enzymatically hydrolyzed into CA and DHPL. Among tested enzymes, only chlorogenate esterase (*A. japonicus*) was able to perform this hydrolysis. This result confirms the hypothesis that RA could be cleaved by a cinnamoyl esterase. The enzymatic hydrolysis of RA to produce CA and DHPL could be an interesting approach for applications in food products. Cinnamoyl esterases have been commonly found in rumen and soil saprophytic microorganisms (*35*) and in bacteria from human and animal intestinal microbiota (*24*). *L. johnsonii* NCC 533 (La1) used in this study is a probiotic strain isolated from the human intestinal microbiota. Complete hydrolysis of RA was achieved with La1 cells, while no cinnamoyl esterase-like activity was identified in both culture and reaction media. Incubation of RA with La1 crude extract resulted in a strong increase of the reaction rate as compared to nonlysed cells. These observations suggest that the enzyme involved in the hydrolysis of RA is presumably intracellular. The identification, purification, and characterization of La1 esterase involved in RA hydrolysis are under investigation. Treatment of rosemary extract with La1 cells showed a complete degradation of RA, while the other key molecules remained intact as shown by LC-MS. The La1 was selected to perform the hydrolysis of RA because this bacterium is known to possess several esterases (genome data). Other lactic acid bacteria were screened and used for their cinnamoyl esterase activity (*26*).

Experiments in the GI tract model (TIM-1) were conducted to see whether the hydrolysis of RA occurs chemically under the conditions of the GI model (e.g., temperature, pH, and bile salts), catalyzed by secreted enzymatic activity (e.g., lipase and pancreatic enzymes), or by selected enzymes and microorganisms. No hydrolysis of RA was observed under the physiological conditions mimicked in the TIM-1 model. The hydrolysis of RA was not catalyzed by secreted enzymatic activity. However, substantial hydrolysis of RA was observed when La1 was added to rosemary extract in the TIM-1 model. These results confirm the hypothesis that RA and probably hydroxycinnamates are degraded by gut microflora before their absorption and are then metabolized in various tissues such as intestine, liver, and kidney.

In conclusion, this is the first time that cinnamoyl esterase-like activity is identified in the probiotic *L. johnsonii* (La1). The results obtained in this study support the hypothesis that RA is degraded by gut microflora before absorption and metabolization.

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