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Honey Varietals Differentially Impact *Bifidobacterium animalis* ssp. *lactis* Survivability in Yogurt through Simulated In Vitro Digestion

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A B S T R A C T

Background: *Bifidobacterium animalis ssp. lactis* DN-173 010/CNCM I-2494 (*B. animalis*) is a probiotic strain commonly added to yogurt. Yogurt and honey are a popular culinary pairing. Honey improves bifidobacteria survival in vitro. However, probiotic survival in yogurt with honey during in vitro digestion has not been investigated.

Objectives: The study aimed to evaluate the effects of different honey varietals and concentrations on *B. animalis* survivability in yogurt through in vitro digestion.

Methods: Yogurt with honey or control-treated samples underwent in vitro simulated oral, gastric, and intestinal digestion. *B. animalis* cells were enumerated on de Man Rogosa and Sharpe (MRS) medium followed by an overlay with a modified selective MRS medium; all underwent anaerobic incubation. *B. animalis* were enumerated predigestion and after oral, gastric, and intestinal digestion. There were 2 study phases: Phase 1 tested 4 honey varietals at 20% wt/wt per 170 g yogurt, and Phase 2 tested 7 dosages of clover honey (20, 14, 10, 9, 8, 6, and 4% wt/wt) per 170 g yogurt.

Results: Similar *B. animalis* counts were observed between all treatments after oral and gastric digestion (<1 Log colony forming units (CFU)/g probiotic reduction). Higher *B. animalis* survivability was observed in yogurt with clover honey after exposure to simulated intestinal fluids (~3.5 Log CFU/g reduction; $P < 0.05$) compared to all control treatments (~5.5 Log CFU/g reduction; $P < 0.05$). Yogurt with 10–20% wt/wt clover honey increased *B. animalis* survivability after simulated in vitro digestion (≤ ~4.7 Log CFU/g survival; $P < 0.05$).

Conclusions: Yogurt with added honey improves probiotic survivability during in vitro digestion. The effective dose of clover honey in yogurt was 10–20% wt/wt per serving (1–2 tablespoons per 170 g yogurt) for increased probiotic survivability during in vitro digestion.

Keywords: honey, in vitro digestion, probiotic, yogurt

Introduction

Yogurt is a fermented dairy product [1] created from spontaneous or induced lactic acid fermentation of milk [2,3]. The microorganisms used to ferment the milk inform their characterization as standard or probiotic yogurts. Conventional yogurts use a standard starter culture (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) [4]. Probiotic yogurts use the required standard culture in addition to supplementation with probiotic strains, typically bifidobacterial and/or lactobacilli [5].

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [6]. Probiotic microbial strains must be *1*) identified genetically (strain-specific), *2*) safe for intended use, *3*) supported by ≥1 human clinical trial, *4*) demonstrate health benefit(s), and *5*) alive in sufficient numbers in the product at an efficacious dose throughout its shelf life [7,8]. Although many fermented foods contain live and active cultures, few qualify as probiotic foods as they do not contain microbes that meet the above conditions [9]. Other factors that prevent certain fermented dairy products from having a probiotic status are their capacity to survive through high-stress environments such as gastrointestinal digestion [10]. An adequate number of viable cells in probiotic yogurts (10^8 cells/g) are necessary to provide

Abbreviations: CFU, colony forming units; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; TPC, total phenolic content.

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strain-specific health benefits, allowing greater opportunity to colonize the intestine [10].

Many commercial probiotic strains are from the bifidobacterium genus [11]. *Bifidobacterium animalis (B. animalis)* is a typical inhabitant of the mammalian colon [12]. *B. animalis* is resistant to acidity, adheres to intestinal mucin, grows in milk, and demonstrates some oxidative stress resistance [12]. Although many single and multi-strain probiotic formulations containing bifidobacteria are available as supplements (e.g., capsules and tablets), *B.* (*animalis) lactis* can be found in foods such as yogurt.

Certain food combinations can optimize nutrient bioavailability (e.g., carrots consumed with oil enhance carotenoid absorption). Also, there is growing evidence that consuming live microbes in the diet helps support health [13]. As yogurt (a source of live microbes and probiotics) is commonly paired with honey, and honey can enhance bifidobacterial survival in vitro [14,15], this study aimed to evaluate the effect of adding 4 different honey varietals (alfalfa, buckwheat, clover, and orange blossom) to commercial yogurt containing probiotic *Bifidobacterium animalis ssp. lactis* DN-173 010/CNCM I-2494 (*B. animalis*), on the probiotic survivability within yogurt during in vitro digestion. We hypothesized that honey would enhance *B. animalis* survival during simulated complete in vitro gastrointestinal digestion.

Methods

Honey characterization

The National Honey Board provided the honey varietals (alfalfa, buckwheat, clover, and orange blossom). Honey from a single production was packaged in 1-pound containers for retail sale in North America and was shipped directly from the supplier to our laboratory and to the analytic laboratory for composition testing. Within 24 h of receipt, samples were stored at –20℃ in airtight 1-pound packages, and aliquots for experimentation were stored at –80°C. Products remained frozen until prepared for use within 10 d of removal from the freezer. The producer used general industry practices to yield honey free of foreign organic matter (heated to <85°C, filtered to 16 microns, and cooled to 51°C for packaging). Honey was tested by the producer to ensure authenticity. The honey varietals originated from different locations: clover was from the Dakotas, alfalfa from Wyoming, orange blossom from Orange Groves, and buckwheat from the Midwest.

Sugar analysis of honey varietals

The sugars from the honey varietals (glucose, fructose, and sucrose) were quantified using high-performance anion exchange chromatography with pulsed amperometry detection (HPAEC-PAD, Dionex ICS-5000; Thermo-Fisher, USA) in conjunction with CarboPac PA1 guard (4 mm x 50 mm; Thermo-Fisher, USA) and analytical (250 mm x 4 mm) column. The sugars were eluted at 25℃ in 10 mM NaOH for 15 min, followed by 100 mM NaOH for 30 min at a 1 mL/min flow rate. Honey samples were diluted in deionized water and filtered through a 0.45 μm nylon filter injection into the chromatographic system. Calibration curves were constructed from pure standards (Sigma-Aldrich, St. Louis, MO) and were used to quantify honey sugars.

Antioxidative and phenolic analysis of honey varietals

The total phenolic content (TPC) in honey was determined by the Folin-Ciocalteu method, as described previously, with minor modifications [16]. Briefly, honey samples were diluted to 30% (wt/vol) solution with distilled water. Twenty-five μL of diluted sample or standard (gallic acid) solution was mixed with 125 μL 0.2 mol/L Folin-Ciocalteu reagent in a 96-well microplate and allowed to react for 10 min at room temperature. Then 125 μL 7.5% (wt/vol) Na_2CO_3 was added and incubated for 60 min at room temperature. The absorbance was measured at 765 nm using a visible–UV microplate kinetic reader (EL 340, Bio-Tek Instruments, Inc., Winooski, VT, USA). TPC was expressed as mg gallic acid equivalents (GAE) per 100 g honey (mg GAE/100 g honey) by using the gallic acid calibration curve.

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was used to assess the antioxidant activity of honey; it was measured according to a previous report [16] with slight modifications. Briefly, 25 μL of 30% (wt/vol) honey sample or standard (Trolox) was mixed with 200 µL of 350 µM DPPH in methanol in a 96-well plate. The mixtures were reacted for 6 h in darkness at room temperature. The absorbance was measured at 517 nm. The DPPH antioxidant activity was expressed as µmol of Trolox equivalents (TE) per 100 g honey (µmol TE/100 g honey) by plotting the percentage of DPPH quenched against the concentration of Trolox.

Ferric-reducing antioxidant power activity of honey was measured following a previously reported procedure [16] with slight modifications. Briefly, 10 μL of 30% (wt/vol) honey sample or standard (ascorbic acid) was allowed to react with 300 μL of ferric-2,4,6-Tris(2-pyridyl)-s-triazine reagent and kept at room temperature for 2 h. The absorbance was read at 593 nm. The ferric-reducing antioxidant power value was expressed as µmol L-ascorbic acid equivalent (AAE) per 100 g honey (µmol AAE/100g honey) using the L-ascorbic acid calibration curve.

The phenolic extract of honey was prepared using acidified aqueous methanol. Briefly, honey samples were diluted to 30% (wt/vol) solution by distilled water and were acidified by formic acid with a final concentration of 1% (vol/vol). Twenty-five mL of acidified honey solution was purified using OASIS HLB polymeric solid phase extraction cartridges (150 mg, Waters, Mississauga, ON, Canada) and eluted with 1% formic acid in methanol (vol/vol). The eluent was used for liquid chromatography-mass spectrometry (LC-MS) analysis. LC-MS/MS analysis was performed using a Thermo Scientific Q-Exactive Orbitrap mass spectrometer equipped with a Vanquish Flex Binary UPLC System. A Kinetex XB-C18 100A column (100 x 4.6 mm, 2.6 μm, Phenomenex Inc.) was used. The binary mobile phase consisted of solvent A (99.9% water (H_2O)/0.1% formic acid) and solvent B (94.9% methanol (MeOH)/5% acetonitrile (ACNI)/0.1% formic acid). The following solvent gradient was used: 0–5 min, 0–12% B; 5–15 min, 12–23% B; 15–30 min, 23–50% B; 0–40 min, 50–80% B; 40–42 min, 80–100% B; 42–45 min, 100% B; 45–46 min, 100–0% B; 46–52 min, 0% B. The column temperature was set at 40℃, the flow rate was set at 0.7 mL/min, and the injection volume was 2 μL; UV peaks were monitored at 280 nm. Mass spectrometry data were collected using both Full MS and dd-MS^2 (data-dependent) modes; negative ionization mode was used, and spray voltage was set at 4.5 kV. FullMS was used for quantification, and DDMS2

(Top N = 10, normalized collision energy [NCE] = 30, intensity threshold = 1.0e5 counts) was used for the tentative identification of the unknown compounds. Data were visualized and analyzed using Thermo FreeStyle 1.7PS2 software.

Organic acid analysis of honey varietals

Honey samples for organic acid analysis were diluted to 1% (wt/vol) solution by 1% (vol/vol) formic acid in distilled water. Further dilution of 0.25% (wt/vol) was prepared to analyze gluconic acid. Samples were filtered by 0.45 μm syringe filters before LC-MS analysis. LC-MS/MS analysis was performed using the same HPLC-MS system as stated above. A Phenomenex Rezex ROA-Organic Acid H^+ (8%) column (150 x 4.6 mm, Phenomenex Inc.) was used. The mobile phase was 0.5% formic acid in water. Separation was achieved using isocratic elution with a flow rate of 0.3 mL/min; the method duration was 7 min. The column temperature was set at 55°C, and the injection volume was 0.5 μL. Mass spectrometry data were collected using the FullMS method, negative ionization mode was used, and the spray voltage was set at 4.0 kV. Data were visualized using Thermo FreeStyle 1.7PS2 software. All analyses were performed in triplicate.

Enzymatic analysis of honey varietals

Amylase activity in the honey varietals was measured by diluting the honey samples to 30% (wt/vol) solution with distilled water. The solution was filtered through a 0.22 μm filter to remove any insoluble materials. Amylase activities were measured using a colorimetric assay kit (Abcam) according to the manufacturer's instructions. Briefly, 50 μL of diluted honey samples or nitrophenol standards were mixed with 100 μL of amylase reaction mix (ethylidene-pNP-G7 and α-glucosidase) in a 96-well plate. Absorbance was measured immediately at 405 nm in a kinetic mode for 60 min at 25°C protected from light. α-Amylase in honey cleaved the substrate ethylidene-pNP-G7 to produce smaller fragments that were eventually modified by α-glucosidase, causing the release of a chromophore that can be measured at 405 nm. The amylase activity was expressed as U/100 g honey by using the nitrophenol calibration curve. One U was defined as the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmol of nitrophenol per minute at pH 7.20 at 25°C.

Diastase activity in the honey varietals was measured by diluting the honey samples to 1% (wt/vol) solution with 0.1M acetate buffer (pH = 5.2). The solution was filtered through a 0.22 μm filter to remove any insoluble materials. Diastase activities were measured using a colorimetric assay kit (Phadebas) according to the manufacturer's instructions. Briefly, 5.0 mL of diluted honey samples or 0.1M acetate buffer using as blank were mixed with 1 Phadebas tablet at 40°C for 30 min. The Phadebas tablet contained 45 mg water-insoluble, cross-linked starch polymer carrying blue dye, which can be hydrolyzed by diastase and generate blue water-soluble fragments. The reaction was stopped by adding 1 mL of 0.5M sodium hydroxide solution. After centrifuging at 1500 × *g*; 5 min, the supernatant was measured in a 1 cm cuvette at 620 nm. Diastase activity was expressed as diastase number (DN) based on the difference of absorption at 620 nm between the sample and blank.

Glucose oxidase activity in the honey varietals was measured by diluting the honey samples to 30% (wt/vol) solution with distilled water. The solution was filtered through a 0.22 μm filter to remove any insoluble materials. Glucose oxidase activities were measured using a colorimetric assay kit (Abcam, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 50 μL of diluted honey samples or glucose oxidase standards were mixed with 50 μL of glucose oxidase reaction mix (glucose, AbRed indicator, and horseradish peroxidase) in a 96-well plate. Absorbance was measured immediately at 570 nm in a kinetic mode for 30 min at 37°C. Glucose oxidase in samples catalyzed the oxidation of β-D-glucose into hydrogen peroxide and D-glucono-1,5-lactone. The produced hydrogen peroxide reacted with the AbRed indicator when catalyzed by horseradish peroxidase to generate the compound, which can be measured at 570 nm. The glucose oxidase activity was expressed as U/100 g honey by using the calibration curve. One U was defined as the amount of glucose oxidase that reacts with 1.0 μmol of glucose/min at 37°C.

Catalase activity was measured by diluting the honey samples to 3.0% (wt/vol) solution with distilled water. The solution was filtered through a 0.22 μm filter to remove any insoluble materials. Catalase activities were measured using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, 20 μL of diluted honey samples or formaldehyde standards were mixed with 100 μL of assay buffer, 30 μL of methanol, and 20 μL of 35.3 mM hydrogen peroxide in a 96-well plate. Catalase in samples catalyzed the peroxidation of methanol to produce formaldehyde after 20 min incubation at room temperature. The formaldehyde was measured calorimetrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) and potassium periodate. The catalase activity was expressed as U/100 g honey by using the formaldehyde calibration curve. One U was defined as the amount of catalase that peroxidizes methanol to generate 1.0 μmol of formaldehyde per minutes at room temperature.

Invertase activity was measured by diluting the honey samples to 30% (wt/vol) solution with distilled water. The solution was filtered through a 0.22 μm filter to remove any insoluble materials. Glucose in honey was removed by centrifuging at 5000 × *g* using an Amicon ultra centrifugal filter device with a molecular weight cut-off of 10,000 (Merck KGaA) ≥7 times. The concentrated protein was collected and dissolved ≤500 μL in the phosphate-buffered saline (PBS) buffer. Invertase activities were measured using a colorimetric assay kit (Abcam, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 25 μL of concentrated protein solution was mixed with 15 μL of assay buffer and 10 μL of sucrose (i.e., invertase substrate). The same sample volume without adding sucrose was prepared simultaneously as a background control. Invertase in honey catalyzed the hydrolysis of sucrose by cleaving its glycosidic bond and forming glucose and fructose. After 20 min of reaction, samples, background controls, and sucrose standards were mixed with the provided enzyme mix and probe to generate a chromogen that can be measured at 570 nm. The absorption of background control was subtracted from the sample to eliminate the influence of residual glucose in a sample. The invertase activity was expressed as mU/100 g honey by using the glucose calibration curve. One mU was defined as the amount of invertase that cleaves sucrose to generate 1.0 nmol of glucose/min at 37°C.

In vitro experimentation phase 1 (comparison of honey varietals)

Honey varietals were stored at –80°C until the experiment began and then thawed in a water bath at 42°C for 30 min until a syrup consistency was observed. The yogurt used in this study was a commercial low-fat vanilla yogurt containing *B. animalis* (Activia). Supplemental Table 1 details the yogurt nutrition information. For the first phase of the in vitro experimentation, probiotic-containing samples were prepared by adding the 4 honey varietals to each yogurt sample to a final concentration of 20% (wt/wt) in the yogurt. Each sample contained 170 g of commercial yogurt plus 42 g of honey or a control component. The controls for the first experiment were an undiluted yogurt, yogurt with added water 20% (wt/vol), and 30.4 g sucrose (isocaloric equivalent to the 42 g honey). After treatment preparation, all samples were stored at 4°C for 72 h to allow *B. animalis* to acclimate to its new yogurt matrix.

Following Brodkorb's protocol with scaled-down modifications [17], enzymatic assays (α-salivary amylase, porcine pepsin, pancreatin, trypsin, and bile salt) were conducted 24 h before the experiment, and activity values were considered valid for 1 wk. For the oral stage of digestion, a spectrophotometric stop reaction was used to calculate the activity of human salivary α-amylase (Sigma-Aldrich, cat. No. 1031) using Equation *1* (b = intercept of linear regression, a = slope of linear regression, X = quantity of amylase powder (mg) added before stopping the reaction): units/mg = $[(A_{540}\ \text{Test} - A_{540}\ \text{Blank}) - b] / (a \times X)$. Each assay replicate had a final amylase activity of 75 U/mL using Equation *1*. The gastric stage of digestion involved assessing pepsin activity using a spectrophotometric stop reaction [17]. First, pepsin was measured to 12,000 U, then diluted to a final concentration of 2000 U/mL using deionized water. Next, 360 U gastric lipase was diluted to 60 U/mL using deionized water [17]. Finally, the activity was calculated using Equation *2* (X = mg quantity of pepsin powder): units/mg = $[(A_{260}\ \text{Test} - A_{260}\ \text{Blank}) \times 1000] / (\Delta t \times X \times 0.001)$. For the intestinal stage of digestion, we conducted 2 assays to measure the trypsin activity in pancreatin using the Brodkorb et al. [17] protocol with modifications. First, 800 U pancreatin was diluted to a final activity of 100 U/mL using deionized water. Next, bile salts were supplied from bovine bile and measured using a commercial kit according to the supplier's protocol (Sigma-Aldrich, cat. No. MAK 309). Finally, trypsin in pancreatin was measured using a kinetic spectrophotometric rate determination method and calculated using Equation *3* (X = quantity of pancreatin used in the final reaction mixture in mg): units/mg = $[(A_{260}\ \text{Test} - A_{260}\ \text{Blank}) \times 1000 \times 3] / (540 \times X)$. Prior testing was performed to establish the amount of strong acid and base needed to maintain the required pH at each stage of digestion.

All yogurt samples underwent in vitro simulation of gastrointestinal digestion using the Brodkorb et al. [17] protocol with modifications. For each stage (oral, gastric, and intestinal), a simulated digestive fluid was prepared and labeled as simulated salivary, gastric, and intestinal fluids, respectively. Each solution was prepared 72 h starting a trial for the experiment and stored at 4°C. For each trial, an aliquot was prewarmed to 37°C the same day of the experiment [17]. The electrolyte solutions used for each were prepared using the following quantities (deionized water was used in all dilutions): 0.5M KCl, 0.5M KH_2PO_4, 1M $NaHCO_3$, 2M NaCl, 0.15M $MgCl_2(H_2O)_6$, 0.5M $(NH_4)_2CO_3$, 6M HCl (as needed to achieve required pH), 0.3M $CaCl_2(H_2O)_2$ [16]. Each simulated fluid (salivary, gastric, and intestinal) was prepared by mixing the following volumes of electrolyte solutions and diluting with water to a final volume of 400 mL to achieve the indicated final mM concentrations and adjusted to pH 7, 3, and 7, respectively. The simulated salivary solution had a pH of 7 and contained 15.1 mM KCl (15.1 mL), 3.7 mM KH_2PO_4 (3.7 mL), 13.6 mM $NaHCO_3$ (6.8 mL), 0.15 mM $MgCl_2(H_2O)_6$ (0.5 mL), 0.06 mM $(NH_4)_2CO_3$ (0.06 mL), 1.1 mM HCl (0.09 mL), 1.5 mM $CaCl_2(H_2O)_2$ (0.025 mL). The simulated gastric fluid had a pH of 3 and contained 6.9 mM KCl (6.9 mL), 0.9 mM KH_2PO_4 (0.9 mL), 25 mM $NaHCO_3$ (12.5 mL), 47.2 mM NaCl (11.8 mL), 0.12 mM $MgCl_2(H_2O)_6$ (0.4 mL), 0.5 mM $(NH_4)_2CO_3$ (0.5 mL), 15.6 mM HCl (1.3 mL), 0.15 mM $CaCl_2(H_2O)_2$ (0.005 mL) [17]. The simulated intestinal fluid had a pH of 7 and contained 6.8 mM KCl (6.8 mL), 0.8 mM KH_2PO_4 (0.8 mL), 42.5 mM $NaHCO_3$ (85 mL), 9.6 mM NaCl (38.4 mL), 0.33 mM $MgCl_2(H_2O)6$ (1.1 mL), 8.4 mM HCl (0.7 mL), 0.6 mM $CaCl_2(H_2O)_2$ (0.04 mL) [17].

The sequential simulated digestion procedure was conducted, as indicated in Table 1, by mixing the sample with the appropriate solutions in a sterile 15 mL polypropylene centrifuge tube followed by incubation at 37°C (Table 1). For all experiments, at 4-time points—predigestion (baseline) and after each stage of digestion (i.e., oral, gastric, and intestinal)—2 mL aliquots were removed and placed in ice to slow and stop the enzymatic activity. The volume ratio of digestive fluid and solution was kept at 1:1 throughout the experiment. Sample aliquots were serially diluted 10-fold using a PBS solution. Enumeration was carried out by spread plating 100 μL of each dilution factor onto separate ~20 mL of solidified de Man Rogosa and Sharpe agar (MRS) supplemented with 0.5 g/L L-cysteine hydrochloride (MRSc; supplemented MRS)

TABLE 1
Sequential simulated in vitro digestion protocol

1. Oral stage (final volume 5 mL, pH 7)
Food-oral fluid ratio 1:1 (vol/vol)
2.5 g yogurt sample
2 mL SSF electrolyte (prewarmed 30 min at 37°C)
12.5 μL $CaCl_2(H_2O)_2$ (final concentration 1.5 mM)
112.5 μL H_2O
375 μL salivary amylase (1000 U/mL) – 375 U – final 75 U/mL
30 min incubation at 37°C
2. Gastric stage (final volume 6 mL, pH 3)
Oral-gastric fluid ratio 1:1 (vol/vol)
3 mL oral stage
2.4 mL SGF electrolyte (prewarmed 30 min at 37°C)
1.5 μL $CaCl_2(H_2O)_2$ (final concentration 0.15 mM)
362.5 μL H_2O
36 μL HCl (6M)
0.2 mL porcine pepsin (60,000 U/mL) – 12,000 U – final 2000 U/mL
120 min incubation at 37°C
3. Intestinal stage (final volume 8 mL, pH 7)
Gastric-intestinal fluid ratio 1:1 (vol/vol)
4 mL gastric stage
1.6 mL SIF electrolyte (prewarmed 30 min at 37°C)
748 μL H_2O
52 μL NaOH (5M)
0.6 mL bile salts (final 10 mM) (prewarmed 30 min at 37°C)
1 mL pancreatin (800 U/mL trypsin) – 800 U – final 100 U/mL
120 min incubation at 37°C

Abbreviations: HCl, hydrochloric acid; pH; simulated gastric fluid (SGF); simulated intestinal fluid (SIF); simulated salivary fluid (SSF).

TABLE 2
Honey composition of the 4 varietals at baseline

	Honey varietals (mean ± SEM)			
	Clover	Buckwheat	Orange	Alfalfa
Sugars (g/100 g honey)				
Fructose	38.5 ± 0.30	37.5 ± 1.01	38.2 ± 0.25	36.6 ± 0.83
Glucose	34.6 ± 0.11^{a}	33.6 ± 0.68 ^{a,b}	31.8 ± 0.21^{b}	32.5 ± 0.50^{b}
Sucrose	0.72 ± 0.01^{a}	0.12 ± 0.01^{b}	0.68 ± 0.01^{a}	0.42 ± 0.01^{c}
Total sugar	73.8 ± 0.42	71.1 ± 1.70	70.7 ± 0.46	69.5 ± 1.34
Phenolic (μg/100 g honey)				
p-Hydroxybenzoic Acid	391 ± 18^{b}	763 ± 28^{a}	354 ± 8.0^{b}	240 ± 13^{c}
Caffeic acid	130 ± 6.0^{c}	190 ± 11^{c}	1300 ± 41^{a}	1200 ± 38^{b}
p-Coumaric acid	330 ± 14^{b}	530 ± 1.9^{a}	160 ± 8.0^{c}	180 ± 8.0^{c}
Trans-ferulic acid	150 ± 5.0^{a}	140 ± 2.0^{b}	70. ± 2.0^{d}	97 ± 3.0^{c}
Abscisic acid	268 ± 10^{b}	244 ± 9^{b}	1070 ± 4^{a}	170 ± 9^{c}
Kaempferol-rutionoside	13 ± 1.0^{c}	88 ± 3.0^{a}	15 ± 1.0^{c}	39 ± 2.0^{b}
Pinobanksin	1100 ± 15^{c}	1300 ± 46^{b}	420 ± 14^{d}	1500 ± 3^{a}
Naringenin	490 ± 8.0^{c}	630 ± 22^{b}	150 ± 7.0^{d}	698 ± 15^{a}
Quercetin	93 ± 2.0^{b}	77 ± 4.0^{c}	110 ± 2.0^{a}	75 ± 3.0^{c}
Kaempferol	690 ± 22^{a}	598 ± 27^{b}	150 ± 4.0^{d}	460 ± 6.0^{c}
Apigenin	280 ± 6.0^{b}	290 ± 12^{b}	72 ± 1.0^{c}	360 ± 9.0^{a}
Pinocembrin	1600 ± 34^{a}	1500 ± 51^{a}	240 ± 4.0^{b}	1600 ± 41^{a}
Biochanin A	320 ± 12^{b}	420 ± 18^{a}	92 ± 2.0^{c}	410 ± 13^{a}
Chrysin	270 ± 10^{c}	370 ± 12^{b}	72 ± 2.0^{d}	510 ± 17^{a}
Galangin	290 ± 8.0^{b}	380 ± 20^{a}	75 ± 3.0^{c}	301 ± 11^{b}
Total phenolics	6400 ± 106^{b}	7500 ± 97^{a}	4300 ± 3.0^{c}	7700 ± 130^{a}
Organic acids (mg/100 g honey)				
Citric acid	5.7 ± 0.11^{c}	6.10 ± 0.10^{b}	11 ± 0.07^{a}	5.3 ± 0.02^{d}
Gluconic acid	370 ± 3.3^{c}	430 ± 5.90^{b}	510 ± 1.8^{a}	503 ± 1.2^{a}
Malic acid	2.8 ± 0.04^{d}	5.20 ± 0.07^{b}	6.40 ± 0.13^{a}	3.5 ± 0.03^{c}
Succinic acid	0.90 ± 0.04^{c}	1.20 ± 0.0^{b}	1.60 ± 0.03^{a}	1.1 ± 0.09^{b,c}
Lactic acid	2.0 ± 0.07^{d}	3.20 ± 0.09^{c}	6.50 ± 0.08^{a}	5.2 ± 0.14^{b}
Total organic acids	380 ± 73^{c}	450 ± 86^{b}	530 ± 99^{a}	520 ± 99^{a}
Total phenolic activity				
TPC (GAE mg/100 g)	36 ± 0.50^{d}	195 ± 2.0^{a}	48 ± 0.50^{b}	43 ± 0.50^{c}
FRAP (AAE mol/100 g)	127 ± 2.1^{c}	571 ± 5.9^{a}	146 ± 1.1^{b}	152 ± 2.0^{b}
DPPH (TE mols/100 g)	83 ± 4.2^{c}	570 ± 18^{a}	142 ± 6.6^{b}	122 ± 5.8^{b}
Total phenolic activity	250 ± 26^{c}	1300 ± 130^{a}	340 ± 32^{b}	320 ± 33^{b}
Enzymes				
Amylase (U/100 g)	22.5 ± 0.58^{b}	19.4 ± 0.81^{c}	17.3 ± 0.61^{d}	27.9 ± 0.84^{a}
Diastase (DN)	7.41 ± 0.10^{b}	5.66 ± 0.09^{d}	6.09 ± 0.07^{c}	8.57 ± 0.06^{a}
Glucose oxidase (U/100 g)	0.72 ± 0.01^{c}	4.86 ± 0.01^{a}	1.04 ± 0.02^{b}	0.47 ± 0.02^{d}
Catalase (U/100 g)	42.7 ± 1.29^{a,b}	35.8 ± 1.77^{d}	46.3 ± 2.45^{a}	40.9 ± 1.02^{b,c}
Invertase (mU/100 g)	12.8 ± 0.14^{c}	30.5 ± 4.99^{b}	39.3 ± 0.75^{a}	27.8 ± 0.86^{b}
Total enzyme	86.1 ± 8.2^{b,c}	96.1 ± 7.03^{b}	110 ± 10^{a}	106 ± 8.18^{a,b}

The superscript (a–d) means with dissimilar letters in a column are significantly different using Tukey's test ($P < 0.05$).
Abbreviations: AAE, L-ascorbic acid equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric-reducing antioxidant power; GAE, gallic acid equivalent; SEM, standard error of the mean; TE, Trolox equivalent; TPC, total phenolic content.

and then incubated for 5 h at 37℃ under anaerobic conditions to allow *B. animalis* cells to recover [18]. Plates were then overlaid with a selective media (~20 mL): MRSc supplemented with lithium chloride (3 g/L) and sodium propionate (4.5 g/L) and incubated at 37℃ for an additional 67 h under anaerobic conditions before the enumeration of *B. animalis* [19]. The incubation time was sufficient to obtain visible colonies on the plates displaying typical *Bifidobacterium* morphology [18]. Additionally, we tested a heat-treated yogurt with the selective medium to ensure that no detectable amounts of bifidobacteria would grow after pasteurization as soon as the internal temperature of the yogurt reached 63℃ and then started a 30 min timer.

All experiments were analyzed independently 3 times with triplicate samples for each time point, and dilutions were plated in triplicate to obtain a mean for each trial.

In vitro experimentation phase 2 (dose-response)

Based on results from Phase 1, which compared the effect of 4 different honey varietals on probiotic survivability, we identified clover honey as having the greatest effect on supporting *B. animalis* survivability in vitro compared to the other 3 honey varietals. Thus, we conducted a second phase of experimentation to assess a dose relationship for clover honey. The dosages used in this second experiment were 0 g (0% wt/wt), 7 g (4% wt/wt), 10.5 g (6% wt/wt), 14 g (8% wt/wt), 17.5 g (9% wt/wt), 21 g (10% wt/wt), 28 g (14% wt/wt), 42 g (20% wt/wt) of honey added to 170 g of yogurt. All samples followed the same procedure as outlined in Phase 1. All experiments were analyzed independently 3 times with triplicate samples for each time point, and dilutions were plated in triplicate to obtain a mean for each trial.

Statistical analyses

Honey characterization data were visualized and analyzed using Thermo FreeStyle 1.7PS2 software. In vitro digestion experiments were analyzed as completely randomly assigned designs using John's Macintosh Project (JMP) 13.1 (SAS Institute Inc.). An analysis of variance was performed to establish the significance of the factor (honey treatments). Honey characterization results (sugar, phenolic, enzymes, and organic acid profiles) and probiotic enumeration results (Log colony forming units (CFU)/g) were evaluated using Tukey's test to determine the significance of mean differences between treatments. Additionally, for probiotic enumeration results, a Dunnett test was used to compare the honey treatment dosages to the control (undiluted yogurt). Spearman's correlation analysis was utilized to assess if there were relations between the honey components and intestinal survivability of *B. animalis*.

Results

For each honey varietal, the concentrations of glucose, fructose, and sucrose were determined as a proportion of the honey (g/100 g varietal), Table 2. Fructose did not differ between the varietals; however, there were differences in sucrose and glucose concentrations among the varietals, with clover honey having the greatest amounts of both sugars compared to the other varietals. The phenolic composition of the honey varietals is reported in Table 2. The clover honey had 10%, 53%, and 36% higher concentrations of *trans*-ferulic acid and 14%, 78%, and 34% higher concentrations of kaempferol compared to the buckwheat, orange, and alfalfa varietals, respectively. Among the enzymes assessed, amylase, diastase, glucose oxidase, and invertase differed among the varietals, Table 2. Clover honey had the lowest amounts of both glucose oxidase and invertase among the varietals. Additional descriptive analysis of the honey varietals sugar properties, physiochemical properties, and other organic acids are included in Supplemental Tables 2–4, respectively.

In the Phase 1 experimentation, each of the 4 honey varietals (alfalfa, buckwheat, clover, and orange blossom) was compared against 3 controls (i.e., undiluted, sucrose, and water). The addition of honey did not affect the survival of *B. animalis* in yogurt during the baseline, oral, or gastric phases. During the intestinal phase, clover honey enhanced the survivability of *B. animalis* in yogurt. (Table 3; Supplemental Figure 1). After intestinal digestion, clover honey demonstrated the least Log CFU/g reduction of *B. animalis* from baseline after complete simulated digestion from baseline (~3.8 Log CFU/g reduction). This reduction in clover was significantly less compared to the controls: sucrose (~5.8 Log CFU/g reduction, $P < 0.05$), water vehicle (~5.9 Log CFU/g reduction, $P < 0.05$), and undiluted (~5.4 Log CFU/g reduction, $P < 0.05$). After intestinal digestion, alfalfa (~4.5 Log CFU/g reduction), buckwheat (~5.5 Log CFU/g reduction), and orange (~5.6 Log CFU/g reduction) had similar reductions in *B. animalis* counts compared to controls, whereas clover honey resulted in higher *B. animalis* counts after exposure to simulated intestinal fluids (~3.8 Log CFU/g reduction) compared to undiluted yogurt, sucrose-added yogurt, and water-added yogurt (~5.5 Log CFU/g reduction, $P < 0.05$).

A correlation matrix was created using Spearman's statistical method for each honey varietal treatment effect on the intestinal survivability of B. *animalis* and its association with the concentrations of the respective honey components reported in Table 2 (Supplemental Figure 2). There were no statistically significant relationships between *B. animalis* survival and the honey components within the respective varietals. Next, a pooled analysis of all the honey varietals was conducted to assess if there was an overall effect of honey on probiotic survivability through in vitro intestinal digestion (Supplemental Figure 3). There were negative associations with B. *animalis* survival and DPPH ($\rho = –0.81$; $P < 0.01$), TPC ($\rho = –0.79$; $P < 0.01$), malic acid ($\rho = –0.68$; $P < 0.05$), succinic acid ($\rho = –0.59$; $P < 0.05$) and glucose oxidase ($\rho = –0.6$; $P < 0.05$). Alternatively, the concentrations of diastase ($\rho = 0.67$; $P < 0.05$) and amylase ($\rho = 0.63$; $P < 0.05$) were positively associated with *B. animalis* intestinal survivability.

Based on the improved survivability of the clover honey after intestinal digestion, further experimentation (Phase 2) was initiated to determine a dose-response relationship between clover honey and *B. animalis* survivability (Table 4; Supplemental Figure 4). The result revealed a similar reduction to that by the same dose of clover honey in the Phase 1 study, from baseline (~4.0 Log CFU/g reduction) at the highest dosage and was again significantly different from the undiluted control (~5.6 Log CFU/g reduction, $P < 0.05$). Furthermore, lower dosages at 28 g (14% wt/wt, ~4.6 Log CFU/g reduction) and 21 g (10% wt/wt, ~4.8 Log CFU/g reduction) were also different from the undiluted control ($P < 0.05$). This indicated a concentration threshold for clover honey that could benefit *B. animalis* survival. Dosages <21 g per 170 g yogurt (10% wt/wt) did not improve *B. animalis* survivability after in vitro intestinal digestion and were not different from the control.

TABLE 3
Effect of different honey varietals on *Bifidobacterium animalis* survivability in yogurt through simulated in vitro digestion

Yogurt treatment	*B. animalis* (log CFU/g) ± SEM			
	Baseline: predigestion	1st stage: oral	2nd stage: gastric	3rd stage: intestinal
Sucrose	8.8 ± 0.17	8.4 ± 0.25	8.3 ± 0.26	3.0 ± 0.50^{A}
Water	8.8 ± 0.11	8.5 ± 0.24	8.3 ± 0.12	2.9 ± 0.52^{A}
Undiluted	8.7 ± 0.59	8.5 ± 0.99	8.4 ± 1.05	$3.4 \pm 0.12^{A,B}$
Orange honey	8.8 ± 0.08	8.4 ± 0.18	8.0 ± 0.13	3.2 ± 0.10^{A}
Alfalfa honey	8.9 ± 0.13	8.5 ± 0.23	8.3 ± 0.08	$4.3 \pm 0.53^{A,B}$
Buckwheat honey	8.8 ± 0.09	8.5 ± 0.18	8.3 ± 0.19	$3.4 \pm 0.63^{A,B}$
Clover honey	8.9 ± 0.17	8.7 ± 0.14	8.3 ± 0.13	5.1 ± 0.81^{B}

Abbreviations: *B. animalis; Bifidobacterium animalis*; CFU, colony forming unit; SEM, standard error of the mean.
The superscript (A, B) means with dissimilar letters in a column are significantly different using Tukey's test ($P < 0.05$) to compare all treatment means to the mean of every other treatment. Means with no letters in a column are not significantly different from each other ($P > 0.05$).

TABLE 4
Effect of clover honey at different dosages on *Bifidobacterium animalis* survivability in yogurt through simulated in vitro digestion

Treatment (g of honey per 170 g serving of commercial yogurt)	*B. animalis* (log CFU/g) ± SEM			
	Baseline: predigestion	1st stage: oral	2nd stage: gastric	3rd stage: intestinal
Undiluted yogurt (0 g)	8.6 ± 0.38	8.4 ± 0.35	8.0 ± 0.38	3.0 ± 0.37^{A,a}
Clover honey (7 g)	8.9 ± 0.19	8.6 ± 0.19	7.6 ± 0.61	2.8 ± 0.72^{A,a}
Clover honey (10.5 g)	8.7 ± 0.21	8.4 ± 0.36	7.8 ± 0.66	2.7 ± 0.63^{A,a}
Clover honey (14 g)	8.4 ± 0.65	8.0 ± 0.52	7.7 ± 0.52	3.3 ± 0.29^{A,B,a}
Clover honey (17.5 g)	8.6 ± 0.71	8.1 ± 0.57	7.7 ± 0.52	3.4 ± 0.27^{A,B,a}
Clover honey (21 g)	8.6 ± 0.48	8.3 ± 0.44	7.9 ± 0.34	3.8 ± 0.26^{A,B,b}
Clover honey (28 g)	8.5 ± 0.57	8.3 ± 0.47	7.8 ± 0.49	3.9 ± 0.44^{A,B,b}
Clover honey (42 g)	8.7 ± 0.21	8.6 ± 0.19	8.2 ± 0.31	4.7 ± 0.65^{B,b}

The superscript (A, B) means with dissimilar letters in a column are significantly different using Tukey's test ($P < 0.05$) to compare all treatment means to the mean of every other treatment.
The superscript (a, b) means with dissimilar letters in a column are significantly different from the control (undiluted yogurt) using Dunnett's test ($P < 0.05$).
Abbreviations: *B. animalis; Bifidobacterium animalis*; CFU, colony forming unit; SEM, standard error of the mean.

Discussion

Herein, we evaluated the effect of pairing 4 honey varietals (alfalfa, buckwheat, clover, and orange blossom) with commercial yogurt on *Bifidobacterium animalis ssp. lactis* DN-173 010/CNCM I-2494 survival through in vitro digestion. Clover honey supported *B. animalis* survival best during simulated in vitro intestinal stages of digestion compared to the other honey varietals and the controls, with a minimum effective dosage of clover honey of 10% (wt/wt) in yogurt. Analysis of the honey nutrient profiles revealed that clover honey had higher concentrations of glucose and sucrose, as well as phenolic compounds kaempferol and *trans*-ferulic acid, compared to other honey varietals. Conversely, clover honey had the lowest concentrations of invertase and glucose oxidase.

Bifidobacterium species metabolize sugars with different degrees of polymerization (mono-, oligo-, and polysaccharides) [20]. The ability to break down various sugars is an evolutionary trait stemming from competing for limited carbohydrate sources in the gastrointestinal tract [20]. Honey is a natural syrup that is, on average, made up of fructose (39%), glucose (31%), maltose (7%), a variety of oligosaccharides (4%), and sucrose (2%) [21]. Honey's saccharide content, acidity, crop year, production area, granulating tendency, color, plant source, and storage conditions vary depending on its origin. These variables contribute to a unique chemical profile for each honey varietal [22]. In addition, the oligosaccharide composition and concentration in honey varietals differ depending on the floral source and honeybee species, as the nectar is digested by the bee via α-D-glucosidase after collection from flowers [21]. Ultimately, honey oligosaccharides can be energy substrates for *Bifidobacterium* [14].

Honey varietals are composed of sugars with different concentrations of fructose, glucose, and sucrose. Our study revealed that all 4 honey varietals varied in fructose and glucose concentrations. Enzymatic activity in the honey also differed, with clover having the lowest invertase activity. Catalase, diastase, and invertase liberate glucose and fructose from oligosaccharides and disaccharides found within honey [23]. Honey ripening is correlated with the invertase activity and sucrose concentration, and low levels of invertase activity indicate honey quality [24]. Glucose oxidase, which was lower in clover and orange honey, also convert glucose into hydrogen peroxide [23]. Our results revealed that clover honey enhanced probiotic survivability through in vitro digestion compared to the other honey varietals and controls. Clover honey contained lower organic acid concentrations (gluconic, malic, and succinic acids) than the other honey varietals. Conversely, clover honey contained greater phenolic concentrations (*trans*-ferulic acid and kaempferol) than the other honey varietals. These viable antioxidative compounds and reducing sugars may have been an active component only when using $\geq$10% clover honey in yogurt, as anything less may not have been sufficient to protect *B. animalis*. Other studies showed that *Bifidobacterium* and lactic acid bacteria strains can transform ferulic acid into p-coumaric acid and caffeic acid, potentially creating a favorable environment for themselves [25]. A lactic acid bacteria strain overexposure to p-coumaric acid induced a stress-induced adaptive response that increased cell surface membrane proteins to counteract the phenolic toxic levels, similarly observed in the gut environment [26]. Follow-up studies are needed to assess if *B. animalis* underwent a similar adaptive response during the 72 h storage period before in vitro digestion and what gene or function was induced because of a similar adaptation described by Reverón et al. [26].

Herein, clover had significantly less gluconic acid than the other varietals. Interestingly, gluconic acid can be utilized by most bifidobacteria strains except for the *B. animalis* species [27]. Thus, the higher gluconic acid concentrations in the orange, alfalfa, and buckwheat varietals may have hindered the probiotic's capacity to adapt to the honey-yogurt food matrix compared to clover, but more studies are needed to test microbial-organic acid interactions. Ultimately, the nutrient profile of clover honey may protect *B. animalis* through gastrointestinal digestion because of the presence of antioxidant phenolic compounds and reducing sugars, both of which can reduce the redox potential and protect anaerobic microbes, like *B. animalis*, from reactive oxygen species [28]. Additionally, different honey varietals will have various antioxidative capacities, which can also be impacted by the gastric and intestinal environments [29]. Future studies should test fractionated clover honey to identify the key fractions responsible for facilitating improved probiotic survivability.

A limitation of the honey nutritional composition profiles is that they only represent the undigested samples. More studies are needed to assess if these phenolic compounds have the same beneficial effect in isolation for *B. animalis* survivability after intestinal digestion. Another limitation was not performing antioxidant assays and measuring the enzymatic activity of the honey varietals after each stage of digestion to assess any shifts from baseline in the antioxidative capacity. As noted in previous studies, the antioxidative capacity of honey is affected by the different environments presented during gastric and intestinal in vitro digestion [29]. Therefore, future studies should track the bioactive activity of honey varietals as they go through different digestion stages. Also, the commercial yogurt studied contained the probiotic *B. animalis;* however, it also included the other live starter cultures that are standard in yogurt fermentation (i.e., *L. bulgaricus*, *Lactococcus lactis (L. lactis)*, and *S. thermophilus*). Thus, there may have been interactions between the probiotic and the starter culture during the baseline preparation, such as commensalism between microbes to break down certain components of the honey varietals that *B. animalis* could, perhaps, not do alone. As our work aimed to investigate the culinary pairing of yogurt and honey, this was beyond the scope of this work. Follow-up studies that aim to understand how *B. animalis* performs outside of the yogurt matrix would benefit by including another control sample that includes yogurt with only *B. animalis*, excluding the other yogurt cultures.

In summary, clover honey mixed with yogurt increases *B. animalis* survivability after complete in vitro gastrointestinal digestion. Furthermore, clover honey effectively improves *B. animalis* survival using 21, 28, and 42 g per 170 g of yogurt. This study demonstrates that a ratio of 2 functional foods (honey:yogurt) supports *B. animalis* survivability through in vitro digestion. Clinical trials are needed to determine if honey and probiotic honey pairing enhance probiotic survival and the associated host benefits.

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Author contributions

The authors' responsibilities were as follows – HDH, MJM, DAA, and LAI-S: designed the research; DAA, LAI-S, TAK, and RC: conducted the experiments; DAA, LAI-S, ARM, TAK, and RC: analyzed the data; DAA and HDH: wrote the first draft of the article; HDH: had primary responsibility for final content, and all authors: read and approved the final manuscript.

Conflict of interest

HDH is a member of the Journal of Nutrition Editorial Board. All other authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.01.010.

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