Comparative effects of two oral rehydration solutions on milk clotting, abomasal luminal pH, and abomasal emptying rate in suckling calves

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ABSTRACT

The effect of adding an oral rehydration therapy (ORT) solution containing bicarbonate, citrate, and acetate to cow's milk on milk clotting, abomasal luminal pH, and abomasal emptying rate was determined in suckling calves. Six male Holstein-Friesian calves with abomasal cannulae, 5 to 13 d of age, were used in a crossover design. Calves were fed 2 L of cow's milk alone, milk with an ORT solution containing a low bicarbonate concentration (25 mmol/L), acetate (12 mmol/L), citrate (12 mmol/L), and glycine (7 mmol/L; group BACG), or milk with an ORT solution containing formate (58 mmol/L) and acetate (15 mmol/L) in randomized order. Clotting of milk was assessed in vivo and in vitro. Abomasal luminal pH was monitored continuously. Abomasal emptying rates were determined by using the change in abomasal luminal pH, acetaminophen absorption, and glucose absorption. The addition of a BACG-ORT solution to cow's milk increased in vitro clotting time by approximately 6 min. All 3 test solutions clotted in vivo by 15 min after the beginning of suckling. The addition of a BACG-ORT solution to cow's milk increased the pH of cow's milk and abomasal fluid by approximately 0.3 pH units. The addition of a BACG-ORT or a formate and acetate-ORT solution to cow's milk increased solution osmolality and slowed the rate of abomasal emptying. We concluded that the addition of BACG-ORT solution to cow's milk did not affect milk clotting in vivo. Recommendations based on the results of in vitro studies that bicarbonate- or citrate-containing ORT solutions should not be fed concurrently with cow's milk do not appear to be relevant to in vivo conditions when 2 L of a low-bicarbonate (25 mmol/L), low-citrate (12 mmol/L) ORT solution is fed.

Key words: calf diarrhea, oral electrolyte solution, strong-ion difference, rehydration

INTRODUCTION

Calf diarrhea is an ongoing problem for beef and dairy producers in the United States. Diarrhea in neonatal calves can lead to dehydration, hyponatremia, hyper D-lactatemia, strong-ion (metabolic) acidosis, hyperkalemia, and impaired cardiovascular and renal function (Constable, 2003; Constable et al., 2005b). Oral rehydration therapy (ORT) solutions provide a practical and inexpensive method for treating mild to moderate strong-ion acidosis and dehydration in neonatal calves that have a suckle reflex. However, there is still uncertainty about the optimal electrolyte concentrations, type of buffer, and type and amount of energy source, as well as the pH and osmolality of the ORT solution (Naylor et al., 1990; Roussel and Kasari, 1990; Constable et al., 2001; Constable, 2003). It is currently believed that the optimal formulation for an ORT solution should include a sodium concentration between 90 and 130 mmol/L, a potassium concentration between 10 and 20 mmol/L, a chloride concentration between 40 and 80 mmol/L, glucose as a source of energy, and 40 to 80 mmol/L of metabolizable (nonbicarbonate) base, such as acetate or propionate, that will lead to systemic alkalization, but not to gastrointestinal tract alkalization (Roussel and Kasari, 1990; Constable et al., 2001; Constable, 2003). The inclusion of an alkalinizing agent is required to treat systemic strong-ion acidosis, which is frequently present in calves with diarrhea (Naylor et al., 1990; Constable et al., 2005a,b). Continued milk feeding to calves showing mild to moderate signs of diarrhea is generally recommended to maintain growth and support the repair of damaged intestinal mucosa (Heath et al., 1989; Garthwaite et al., 1994). This is because the energy content in ORT solutions is inadequate to meet the needs of the calf, even in high-glucose ORT solutions (Constable et al., 2001).

Diakur Plus (Diaque, Benfital Plus, Boehringer Ingelheim Vetmedica, Copenhagen, Denmark) is an ORT solution that contains sodium, potassium, chloride, and 4 agents (glucose, acetate, citrate, and glycine) that facilitate sodium and free-water absorption in the small intestine of calves (Bywater, 1980; Demigné et al.,

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1983). The commercially available ORT solution contains a low concentration of bicarbonate (25 mmol/L) and 2 metabolizable agents (acetate and citrate) that will result in systemic alkalinization. The ORT solution also contains a lecithin-coated pectin fiber that decreases the proliferation of *Escherichia coli* and *Salmonella* spp. (Faris et al., 1981; Bachman and Larsen, 1989). The citrate concentration (12 mmol/L) of the ORT solution is similar to that (15.5 mmol/L; 4 mg/mL) shown to inhibit growth of *Clostridium perfringens* (Skrivanova et al., 2006). The ORT solution therefore inhibits the growth of 3 important enteric pathogens of suckling calves.

It is widely believed that bicarbonate- or citrate-containing ORT solutions interfere with normal clot formation in the abomasum of milk-fed calves (Heath et al., 1989; Nappert and Spennick, 2003). However, we were unable to find any in vivo studies demonstrating that bicarbonate- or citrate-containing ORT solutions inhibited milk clotting in the abomasum of the suckling calf. The results of 3 in vitro studies indicated that bicarbonate- or citrate-containing ORT solutions markedly prolonged or inhibited milk-clotting time when mixed with whole milk (Bywater, 1980; Naylor, 1992; Nappert and Spennick, 2003), whereas ORT solutions containing acetate or propionate clotted within 10 min (Nappert and Spennick, 2003). Accordingly, the first aim of the study reported here was to determine whether suckling an ORT solution containing a low concentration of bicarbonate (25 mmol/L) and citrate (12 mmol/L) in cow's milk interferes with the clotting of milk in the abomasum of the calf.

Orally administered bicarbonate is an effective systemic alkalinizing agent that does not require metabolism to exert its effect. However, bicarbonate alkalinizes the abomasum of the calf to a greater degree than does suckling a metabolizable base such as acetate (Nouri and Constable, 2006; Marshall et al., 2008), and excessive gastrointestinal alkalinization can facilitate the growth of enteric pathogens (Nouri and Constable, 2006; Marshall et al., 2008). Bicarbonate-containing ORT solutions therefore have the potential to promote the survival and growth of bacterial enteric pathogens such as enterotoxigenic *E. coli* and *Salmonella* spp. The second aim of the study reported here was therefore to determine whether suckling an ORT solution containing bicarbonate, acetate, and citrate in cow's milk would produce a sustained elevation of abomasal luminal pH compared with suckling cow's milk alone.

The rate of abomasal emptying influences the rate at which ingesta are delivered to the small intestine. In the case of an ORT solution, the small intestine is the major site of fluid absorption, and the rate of abomasal emptying is therefore an important determinant of the

rate of rehydration in a dehydrated calf with diarrhea. The volume and caloric content of an ingested fluid meal are the most important determinants of gastric emptying rate (Sen et al., 2006). Other important determinants of emptying rate are the type of protein or fat, osmolality, and duodenal pH, with a solution osmolality of 600 mOsm/kg or a luminal pH of <2.0 or >10.0 decreasing the abomasal emptying rate in suckling calves (Bell et al., 1981; Sen et al., 2006). Because the theoretical osmolality of the commercially available ORT solution containing bicarbonate, acetate, and citrate in cow's milk is 669 mOsm/kg (Table 1), we hypothesized that suckling this solution would result in a slower rate of abomasal emptying than that after suckling cow's milk alone. The third aim of the study reported here was therefore to determine whether suckling an ORT solution containing bicarbonate, acetate, citrate, and glycine in cow's milk would slow the rate of abomasal emptying, as assessed by acetaminophen absorption, glucose absorption, and the change in abomasal luminal pH. To assist in interpreting the results, we studied the effect of suckling another commercially available ORT solution that did not contain bicarbonate (Rehydion Gel, Orion Pharma AS Animal Health, Ceva, Oslo Norway) in cow's milk on in vivo milk clotting, abomasal luminal pH, and abomasal emptying rate.

MATERIALS AND METHODS

Animals and Instrumentation

Seven healthy male Holstein-Friesian calves were obtained from the Purdue University Dairy Center on d 2 to 3 of life. The calves had been fed 4 L of first-milking colostrum within 4 h of birth and an additional 2 L of colostrum 10 to 12 h later. Calves were kept unrestrained in an individual stall in a climate-controlled environment. Cow's milk (approximate characteristics: fat, 3.5%; protein, 3.4%; sugar 5.6%; TS, 12.5%) was obtained from the bulk milk tank and stored at 4°C for up to 4 d before being fed to calves. Cow's milk was fed in a bottle with a nipple twice a day (60 mL/kg of BW) at approximately 12-h intervals after being warmed to approximately 38°C by placing it in a bucket of warm water. Calves had access to fresh water and ad libitum access to calf starter ration (CP, 18%; crude fat, 3%; crude fiber, 6%; ADF, 10%) at all times except for a 10-h period immediately before anesthesia was induced for surgical placement of abomasal cannulae. The study was approved by our Institutional Animal Care and Use Committee.

A 16-gauge catheter (Angiocath, Becton Dickinson, Franklin, NJ) was placed aseptically in the jugular vein at least 10 h before surgery on d 3 or 4 of life. Ceftiofur

Table 1. Components of 2 oral rehydration therapy (ORT) solutions containing bicarbonate, acetate, citrate, and glycine, or formate and acetate in cow's milk¹

Ingredient	Bicarbonate, acetate, citrate, and glycine ORT in water	Formate and acetate ORT in water	Holstein-Friesian cow's milk ²	Bicarbonate, acetate, citrate, and glycine ORT in cow's milk (theoretical)	Bicarbonate, acetate, citrate, and glycine ORT in cow's milk (measured)	Formate and acetate ORT in cow's milk (theoretical)	Formate and acetate ORT in cow's milk (measured)
Cation							
Sodium, mmol/L	90	110	18	108	88.3 ± 7.1	128	82.5 ± 37.7
Potassium, mmol/L	15	27	36	51	53.3 ± 1.0	63	58.0 ± 8.3
Calcium, mmol/L	0	0	31	31	25.7 ± 2.4	31	30.0 ± 1.2
Anion							
Chloride, mmol/L	55	51	16	71	69.8 ± 5.4	67	69.7 ± 26.1
Bicarbonate, mmol/L	25	0	0	25	8.9 ± 3.9	0	0 ± 0
Citrate, mmol/L	12	0	9	21	ND ³	9	ND
Acetate, mmol/L	12	15	0	12	ND	15	ND
Formate, mmol/L	0	58	0	0	ND	58	ND
Phosphate, mmol/L	0	0	37	37	ND	37	ND
Other							
Glucose, mmol/L	161	24	146 ⁴	307	ND	170	ND
Glycine, mmol/L	7	0	0	7	ND	0	ND
Total osmolality, mOsm/kg	377	285	292	669	619 ± 37	577	535 ± 30
Effective strong ion difference, ⁵ mEq/L	50	86	38	88	ND	124	ND

¹Data are expressed as mean or mean ± SD.²From Davies and White (1959) and Cerbulis and Farrell (1976).³ND = not determined.⁴Maximum theoretical glucose concentration derived from the breakdown of the disaccharide lactose (146 mmol/L) into glucose and galactose.⁵Effective strong ion difference = $[Na^+] + [K^+] - [Cl^-]$.

(1.0 mg/kg of BW, i.m., Excenel, Pfizer, Kalamazoo, MI) was administered immediately before surgery and daily for 2 d after surgery. Anesthesia was induced with diazepam (0.25 mg/kg of BW, i.v.) and ketamine (4 mg/kg of BW, i.v.). Calves were endotracheally intubated and allowed to breathe isoflurane (1 to 2%) in 100% oxygen by using a semiclosed rebreathing circuit. Calves were placed in left lateral recumbency on a heating pad and the right flank and paramedian region were prepared for aseptic surgery. Once a surgical plane of anesthesia was obtained, abomasal cannulae (Percutaneous Endoscopic Gastrostomy Kit, MILA Instruments Inc., Florence, KY) were surgically placed as described previously (Ahmed et al., 2001). One cannula was placed in the abomasal body to permit continuous measurement of luminal pH, and the second cannula was placed in the pyloric antrum to permit collection of abomasal fluid for examination of milk clotting and biochemical analysis of abomasal fluid. Flunixin meglumine (Banamine, Schering-Plough Animal Health Corp., Union, NJ; 0.5 mg/kg, i.v.) was administered twice after surgery, at 12-h intervals, for postoperative analgesia.

Venous catheters were replaced when needed in the jugular (16 g) or saphenous vein (18 g) at least 12 h before a study. Venous catheterization of the jugular

vein was performed after sedation with xylazine (0.2 mg/kg of BW, i.m.), aseptic preparation of the skin, and injection of 1 mL of lidocaine under the skin over the vein to be catheterized. A short incision (<1 cm in length) was made in the skin with a scalpel blade to assist in catheter placement, and a catheter was placed in the vein. Venous catheterization of the saphenous vein was performed after aseptic preparation of the skin and injection of 1 mL of lidocaine under the skin over the vein to be catheterized. An extension set was attached to the catheter, and the catheter and extension set were secured to the neck (jugular vein) or hind leg (saphenous vein). Venous catheters were flushed every 8 to 16 h with heparinized 0.9% NaCl solution (40 U of heparin/mL).

Experimental Protocol

The calf was weighed in the morning immediately before each study began. Studies were conducted between d 5 and 13 of age, at least 2 d after surgical cannulation, and at least 10 h after the previous feeding of milk replacer. A collar was placed around the neck of the calf and a chain was connected to the collar in such a manner that the calf could sit and stand easily and move with freedom around the stall but could not turn

in a complete circle. Ten milliliters of distilled water was injected once into each cannula to clean the cannula and dislodge any adherent milk clots.

A flexible miniature glass pH electrode (M3 internal reference glass pH electrode, Medical Instruments Corporation, Solothurn, Switzerland) was calibrated immediately before insertion against reference buffer solutions of pH 7.02 and 1.68 at 20°C and placed into the abomasal lumen via the cannula in the abomasal body as described previously (Ahmed et al., 2001). The pH electrode was connected to a pH meter (Cole-Parmer pH/mV/Rel mV/°C Bench Top Meter, Cole-Parmer Instrument Co., Vernon Hills, IL) and the analog output was digitized at 1 Hz. Digitized data were stored and analyzed offline by using commercially available software (Windaq, DATAQ Instruments, Akron, OH) on a personal computer.

Treatment order was randomly assigned, with a minimum washout period of at least 48 h between each treatment. Calves suckled 2 L of each of the following 3 solutions at approximately 38°C twice at 12-h intervals from a nipple bottle: cow's milk (group **M**); a low bicarbonate-, acetate-, citrate-, and glycine-containing ORT solution mixed directly into 2 L of cow's milk, thereby making an ORT in milk emulsion (100 g of Diakur Plus powder mixed into 2 L of cow's milk; group **BACG**); a formate- and acetate-containing ORT solution in cow's milk (40 mL of Rehydion Gel mixed with 2 L of cow's milk; group **FA**). Diakur Plus is reported to contain 57 g of glucose, 15 g of citrus pulp, 6 g of lecithin, 5 g of sodium chloride, 4 g of sodium bicarbonate, 3 g of silicon dioxide, 2 g of sodium acetate, 2 g of sodium citrate, 2 g of potassium chloride, 1 g of glycine, and 3 g of other compounds per 100 g of powder (product insert). Rehydion Gel is reported to contain 15.0 g of sodium salts as sodium chloride, sodium acetate, sodium diacetate, and sodium formate; 3.5 g of potassium chloride; and 0.2 MJ of energy per 40 mL of solution (www.ceva.uk.com). The concentration and calculated osmolality of individual ORT components when added to water for Diakur Plus and Rehydion Gel are presented in Table 1. The group BACG and FA solutions were fed within 1 min after mixing to avoid sedimentation. Acetaminophen (*N*-acetyl-4-aminophenol; Sigma Aldrich, St. Louis, MO; 50 mg/kg) was mixed with 2 L of the test solution for the first feeding, but was not added to the second feeding.

Abomasal luminal pH was monitored continuously for at least 24 h beginning at least 15 min before the morning feeding. Aspiration of up to 10 mL of abomasal fluid was attempted through the cannula in the pyloric antrum immediately before suckling (time = 0 min) and at 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h after the beginning of suckling. Five milliliters of the abomasal

fluid sample was used for in vivo and in vitro assessment of clotting, whereas another 5-mL fluid sample was frozen and stored at -20°C for subsequent biochemical analysis. If abomasal fluid was not obtained from the cannula immediately before suckling, a small volume of air (10 mL) was injected into the cannula in an attempt to dislodge a milk clot from the cannula lumen. If this procedure of filling the cannula with air failed to dislodge a milk clot, then it was repeated up to 2 times.

Venous blood samples for determination of plasma acetaminophen concentration were obtained at 0 min (immediately before suckling), and at 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, 420, 480, and 720 min after the beginning of suckling the test solution. These sampling times were selected in an attempt to have 6 data points before and after the anticipated time of maximal acetaminophen concentration to facilitate nonlinear regression analysis. Blood samples were collected into 6-mL tubes containing sodium fluoride and potassium oxalate, centrifuged at $1,000 \times g$ for 15 min at room temperature, and 3 mL of plasma was collected and stored at -20°C until analyzed for plasma acetaminophen concentration by using a spectrophotometric technique, as described previously (Marshall et al., 2005). Plasma samples were also stored at -20°C for biochemical analysis.

At the end of the 24-h pH recording period, the pH electrode was removed from the calf and placed sequentially in buffer solutions of 7.02 and 1.68 to determine the extent of pH electrode drift over the 24-h recording period. A fecal sample was obtained from the rectum to determine fecal DM percentage and was stored at -20°C. The calf was then fed its allotted volume of cow's milk (6% of BW twice daily).

At least 48 h after the last feeding of a test solution, healthy calves were randomly assigned to be fed 1 of the 3 test solutions described above. Calves were euthanatized 15 min after the beginning of suckling by intravenous administration of an overdose of sodium pentobarbital (60 mg/kg). The abdomen was rapidly incised, the abomasum was identified, and the nature and extent of the milk clot in the abomasum was characterized.

In Vitro and In Vivo Assessment of Milk Clotting

The in vitro clotting time of each test solution was determined by using bovine chymosin and a modification (Nappert and Spennick, 2003) of a technique described previously (Naylor, 1992). Briefly, this involved the addition of chymosin (Sigma Aldrich; 0.1 unit/mL of test solution), made fresh weekly and stored at 4°C, to 5 mL of solution at 37°C. Clotting time was determined at 2,

4, 6, 8, 10, 12, or 15 min after the addition of chymosin by removing the tube from the water bath and shining a strong light source on the polystyrene tube containing abomasal fluid. The tube was held against a dark background and examined for the presence of flakes, which indicated clot formation. The tube was shaken after each examination and returned to the water bath. The *in vitro* clotting time was defined as the time when flakes became visible, with the result being recorded as 2, 4, 6, 8, 10, 12, or 15 min or as no clot being observed. Samples with no observed clotting after 15 min were classified as nonclotting. To characterize the size of clot formation, after 15 min all samples were poured through a series of 4 sieves of 1.00, 0.71, 0.50, and 0.25 mm, respectively, and the presence or absence of clot retention was noted on each sieve.

The *in vivo* clotting of ingested test solutions was categorized as present or absent by shining a strong light source on a glass tube containing 5 mL of abomasal fluid. The tube was held against a dark background and visually inspected for the presence of flakes (which indicated clot formation) or the absence of flakes. For samples with clot formation, the size of the clot was characterized by pouring the sample through a series of 4 sieves of 1.00, 0.71, 0.50, and 0.25 mm, respectively, and the presence or absence of clot retention was noted on each sieve.

Acid Titration and Buffer Value of Test Solutions

Two liters of the 3 test solutions [milk (group M); milk with an ORT solution containing bicarbonate, acetate, citrate, and glycine (group BACG); milk with an ORT solution containing formate and acetate (group FA)] were prepared as described previously. One hundred milliliters of each test solution was placed in a 250-mL Erlenmeyer flask, warmed to 38°C in a water bath, and the pH of the test solution measured. A 0.5 *N* HCl solution was also warmed to 38°C in a water bath. The test solution was vigorously and continuously stirred by using a magnetic stirrer to minimize protein precipitation as acid titration proceeded through the isoelectric region for CN (pH 4.5; Lucey et al., 1993). Every 30 s, 0.4 mL of 0.5 *N* HCl was added to the test solution (Lucey et al., 1993). The resultant pH was recorded at the end of each 30-s period, and titration was continued until pH was <2.0. Titration was performed in triplicate, the mean pH was calculated, and the mean pH value was smoothed by applying a 5-point moving average.

A plot was developed of smoothed mean pH versus millimoles of HCl added. From the values used in this plot, the buffer value [$\beta = \text{dB/dpH}$; (mol/L)/pH unit] was calculated at the midpoint of the pH range involved

as follows: $\beta = [(\text{milliliters of HCl added to produce pH change}) \times (\text{normality of HCl in millimoles per milliliter})] / [\text{average volume in milliliters of sample over the range involved}] \times (\text{pH change produced})$, so that $\beta = [(0.4 \text{ mL}) \times (0.5 \text{ mmol/mL})] / [(100 \text{ mL} + \text{total volume added in milliliters} - 0.2 \text{ mL}) \times (\text{pH change})]$. The calculated buffer value was smoothed by applying a 3-point moving average, and β was plotted against the pH value at the midpoint of the pH range involved.

Data Analysis

Abomasal Luminal pH. The lowest pH value for each 60-s interval was used as the pH value for that minute; this procedure prevented the inclusion of transient high pH values caused by the pH electrode contacting the abomasal mucosa or a clot. The raw luminal pH-time curves were examined by an investigator (P. D. C.) masked to treatment group. Time points with biologically implausible values for pH, caused by the presence of a milk clot around the pH electrode, were deleted from data analysis.

The median preprandial pH (from time -15 to 0 min), maximum pH after suckling, minimum pH after suckling, and median postsuckling pH were determined. The pH return time was calculated as the time required for luminal pH to return to within 1.0 pH unit of the mean preprandial pH value. A cut point of 1.0 pH units provided the best method for describing the abomasal emptying rate in suckling calves (Marshall et al., 2004).

Acetaminophen Absorption. Acetaminophen was administered to each test solution to evaluate the abomasal emptying rate. Acetaminophen is a widely used oral analgesic and antipyretic drug in humans, and acetaminophen absorption provides an accurate method of determining the emptying rate of liquid-phase meals in calves (Marshall et al., 2005). When administered orally, acetaminophen is absorbed in the small intestine with the rate-limiting step for absorption being the rate of gastric emptying in animals with normal small intestinal function. Because the apparent rate of absorption is faster than the rate of elimination in suckling calves (Marshall et al., 2005), the maximal acetaminophen concentration and time to maximal acetaminophen concentration after oral ingestion are primarily dependent on the rate of abomasal emptying.

The actual maximum observed plasma concentration (C_{max}) and time of actual maximum observed plasma concentration (T_{max}) were obtained from a plot of the plasma acetaminophen concentration - time data. The first derivative of a modified power exponential formula was used to describe the plasma acetaminophen concentration-time relationship:

$$C(t) = m \cdot k \cdot \beta \cdot e^{-k \cdot t} (1 - e^{-k \cdot t})^{\beta-1},$$

where $C(t)$ is the acetaminophen concentration in plasma ($\mu\text{g/mL}$) at time t in minutes, and m , k , and β are constants; m is the total cumulative acetaminophen recovery when time is infinite. This model provided the best method for describing the acetaminophen absorption curve in suckling calves (Marshall et al., 2005). The time to calculate $C_{\max}$ (model $T_{\max}$) was obtained as model $T_{\max} = \ln(\beta)/k$, and the calculated value for model $C_{\max}$ was determined by applying the values for m , k , β , and $t = \text{model } T_{\max}$ to the cumulative dose curve.

Glucose Absorption Curve. Plasma glucose concentration was determined by using a hexokinase, glucose-6-phosphate dehydrogenase method and an automatic analyzer (Hitachi 917 Automatic Analyzer, Hitachi, Tokyo, Japan). The maximum observed plasma concentration (actual $C_{\max}$) and time of maximum observed plasma concentration (actual $T_{\max}$) were obtained from a plot of the plasma glucose concentration-time data. The area under the plasma glucose concentration-time curve was calculated from 0 to 6 h by using the trapezoid method to provide a crude index of the amount of glucose absorbed for each treatment.

Calculated Change in Plasma Volume. Plasma total protein concentration was determined by using a bromocresol green assay performed on an automatic analyzer (Hitachi 917 Automatic Analyzer, Hitachi). The change in plasma volume at time i was calculated from the plasma protein concentration at time = 0 min (PP_0) and the plasma protein concentration at time i (PP_i), whereby the percentage change in plasma volume at time $i = (PP_0 - PP_i) \times 100/PP_i$ (Nouri and Constable, 2006).

Abomasal Fluid and Plasma Biochemical Analysis. Abomasal fluid osmolality was determined by using freezing point depression (Advanced 3MO, Advanced Instruments Inc., Norwood, MS). Ion-specific potentiometry was used to measure abomasal fluid sodium, chloride, and potassium concentrations after appropriate dilutions (Hitachi 917 Automatic Analyzer, Hitachi). The abomasal fluid calcium concentration was measured by using a colorimetric method based on formation of a calcium-cresolphalein complex (Hitachi 917 Automatic Analyzer, Hitachi) after appropriate dilutions. The total carbon dioxide concentrations in plasma and in the test solutions were determined by using an automatic analyzer (Hitachi 917 Automatic Analyzer, Hitachi). Measured strong-ion difference (SID) was calculated as $\text{SID} = (\text{Na}^+ + \text{K}^+ + \text{Ca}^{2+}) - \text{Cl}^-$.

Fecal DM Percentage. The fecal sample was thawed at room temperature and dried at 95°C to a constant

weight. The fecal DM percentage was calculated from the wet weight and dry weight values.

Statistical Analysis

Data were retained for analysis from those calves that remained healthy and suckled the 3 test solutions aggressively. Data were expressed as means and standard deviations, and $P < 0.05$ was considered significant. A statistical software program (SAS 9.1, SAS Institute, Cary, NC) was used for all statistical analyses.

The acetaminophen absorption curve was fit by using nonlinear regression (PROC NLIN, SAS Institute). The adequacy of model fit was assessed by visual examination of plots of observed versus predicted concentrations and by examination of residual plots.

In vitro clotting time and solution pH; osmolality; and sodium, potassium, calcium, and chloride concentrations for the 3 test solutions were compared by ANOVA (PROC MIXED, SAS Institute) and a compound symmetry structure. Abomasal luminal pH and abomasal emptying rate indices were compared by using repeated measures ANOVA (PROC MIXED) and a compound symmetry structure. The change in plasma glucose, total protein, and total carbon dioxide concentrations, percentage change in plasma volume, and fecal DM percentage were compared by using repeated measures ANOVA (PROC MIXED) and a first-order autoregressive structure. The effects of treatment, time, or their interaction were considered fixed and animal was considered random. The 2 primary variables of interest examined to determine the effect of treatment on clotting were the in vitro time to clot formation and the in vivo presence or absence of clots. The 2 primary variables of interest that examined the effect of treatment on abomasal luminal pH were maximal luminal pH and median 24-h luminal pH. The 3 primary variables of interest that examined the effect of treatment on abomasal emptying rate were acetaminophen model $T_{\max}$, time for postprandial pH to decrease to 1.0 pH units above the median preprandial pH value, and glucose $T_{\max}$. The primary variable of interest that examined the effect of treatment on extracellular fluid shifts was the calculated change in plasma volume.

RESULTS

Calves

One calf suckled well for the first 2 treatments, then began to exhibit clinical signs of respiratory disease that were accompanied by a fever and reduced suckle. This calf was euthanatized before it suckled the third

Table 2. Summary of physical examination findings on the day of each study, in vitro clotting time, and results for the 3 methods for measuring abomasal emptying rate in 6 Holstein-Friesian calves suckling 2 L of cow's milk (group M); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in 2 L of cow's milk (group BACG); or a formate- and acetate-containing ORT solution in 2 L of cow's milk (group FA) containing acetaminophen (50 mg/kg of BW)¹

Factor	Group M	Group BACG	Group FA
Physical examination findings			
Age, d	10.0 ± 2.8	8.5 ± 2.6	10.5 ± 3.4
Temperature, °F	103.0 ± 0.7	102.7 ± 0.5	103.1 ± 0.7
Heart rate, beats/min	130 ± 7	125 ± 12	129 ± 9
Respiratory rate, breaths/min	44 ± 5	48 ± 5	46 ± 4
BW, kg	50 ± 4	50 ± 5	51 ± 5
Suckle time 0 h, s	130 ± 55	157 ± 21	139 ± 42
Suckle time 12 h, s	125 ± 45	143 ± 43	138 ± 52
Milk clotting			
In vitro clotting time, min	4.4 ± 0.9	10.8 ± 2.4*	4.0 ± 1.4
Abomasal luminal pH			
Median preprandial pH	1.28 ± 0.28	1.29 ± 0.34	1.29 ± 0.29
Maximum postsuckling pH	5.42 ± 0.79	6.20 ± 0.81*	5.69 ± 0.66*
Minimum postsuckling pH	0.89 ± 0.13	0.95 ± 0.26	0.99 ± 0.23
Median postsuckling pH	1.48 ± 0.13	1.85 ± 0.54	1.67 ± 0.29
pH return time, min	144 ± 33	236 ± 29*	194 ± 34*
Acetaminophen absorption ²			
Actual C _{max} , µg/mL	26.4 ± 3.7	25.3 ± 1.9	23.0 ± 3.4
Actual T _{max} , min	160 ± 31	260 ± 49*	190 ± 70
Model C _{max} , µg/mL	23.8 ± 3.6	24.2 ± 1.2	21.3 ± 3.7
Model T _{max} , min	187 ± 40	236 ± 47*	207 ± 34*
Glucose absorption ²			
Actual C _{max} , mg/dL	126 ± 18	164 ± 63*	131 ± 19
Actual T _{max} , min	104 ± 80	143 ± 53	140 ± 116
Area under the glucose concentration-time curve for the first 6 h, mg/dL × h	608 ± 39	733 ± 112*	613 ± 36

¹Data are expressed as mean ± SD. Mean values for physical examination findings on the morning of the study is provided.

²C_{max} = actual maximum observed plasma concentration; T_{max} = time of actual maximum observed plasma concentration.

*Mean is significantly different from the mean value for cow's milk.

solution, and results from the first 2 studies for this calf were not included in the analysis.

Six calves remained healthy and suckled their milk aggressively for all 3 treatments, which were administered when the calves ranged in age from 6 to 15 d old. There were no differences in the age, rectal temperature, heart rate, respiratory rate, and BW, as well as the suckle time at the 0- and 12-h feedings, when calves were fed the 3 treatments (Table 2). The fecal DM percentage at 24 h after the beginning of suckling each treatment was similar for all 3 treatments: group M, 27.1 ± 2.3%; group BACG, 27.0 ± 3.0%; group FA, 26.5 ± 2.5%.

In Vitro and In Vivo Assessment of Milk Clotting

All BACG-ORT solutions in cow's milk clotted in vitro within 15 min (range 5 to 15 min). The mean in vitro clotting time for the BACG-ORT solution in cow's milk was 6.4 min longer ($P = 0.0006$) than that of cow's milk alone (Table 1). The in vitro clotting time of the FA-ORT solution in cow's milk was similar to that of cow's milk alone.

All fluid samples obtained from the cannula in the pyloric antrum after suckling indicated that clotting

had occurred, as evidenced by the presence of visible flakes that were retained on the 1-mm sieve. However, because of the presence of large clots in the abomasum of some calves, the first abomasal fluid samples were obtained at a median time of 30 min for cow's milk alone (range 15 to 120 min), 15 min for the BACG-ORT solution in cow's milk (range 15 to 15 min), and 15 min for the FA-ORT solution in cow's milk (range 15 to 30 min). The in vivo results were therefore consistent with in vitro findings indicating that all test solutions clotted within 15 min.

One of the 6 calves did not consistently suckle aggressively after the last treatment trial, and the effect of suckling the test solution on the nature and extent of the milk clot in the abomasum was not characterized by necropsy examination of this calf. For the remaining 5 calves, 2 suckled the BACG-ORT solution in 2 L of cow's milk, 2 suckled the FA-ORT solution in 2 L of cow's milk, and 1 suckled 2 L of cow's milk alone, with the calves being euthanatized 15 min after the beginning of suckling. Visual inspection of the abomasal contents (at approximately 18 to 20 min after the beginning of suckling) indicated that extensive milk clotting was present in all 5 calves.

Figure 1. Acid-titration curves (top panel) and the relationship between buffer value and solution pH (bottom panel) for 3 test solutions: cow's milk (group M, ○); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in cow's milk (group BACG, ●); and a formate- and acetate-containing ORT solution in cow's milk (group FA, ▲). Note the increased buffer value for the 3 solutions over a pH range of 4.4 to 5.4, the increased buffer value for groups BACG and FA at a pH of approximately 4.85 (the pK_a of acetic acid), and the increased buffer value for group FA at a pH of approximately 3.6 (the pK_a of formic acid).

Acid Titration Curve and Buffer Value of Test Solutions

The mean solution pH at 38°C was 6.53 ± 0.02 for cow's milk, 6.80 ± 0.10 for the BACG-ORT solution in cow's milk, and 6.25 ± 0.05 for the FA-ORT solution in cow's milk (Figure 1). In other words, the addition of a BACG-ORT solution slightly increased the pH of cow's milk, whereas the addition of an FA-ORT solution decreased the pH of cow's milk. The acid titration curve for cow's milk and the BACG-ORT solution in cow's milk were approximately linear over the pH range studied (Figure 1), whereas the acid titration curve for the FA-ORT solution in cow's milk was curvilinear.

The buffer value of cow's milk was increased over the pH range of 4.4 to 5.4 (Figure 1). Addition of the BACG-ORT solution to cow's milk increased the buffer value above that of cow's milk alone at a pH range of 5.4 to 6.5. Addition of the BACG-ORT or FA-ORT so-

lution to cow's milk increased the buffer value at a pH range of 4.6 to 5.1, with a peak value at approximately 4.85. Addition of the FA-ORT solution to cow's milk increased the buffer capacity over a pH range of 2.8 to 4.2.

Abomasal Luminal pH

Mean electrode drift during the 24-h recording period was 0.00 pH units (range -0.13 to $+0.09$) for buffer pH of 7.02 and -0.02 pH units (range -0.09 to $+0.07$) for a buffer pH of 1.68. Raw pH values were therefore used for statistical analysis because of the minimal drift. The percentage of the total number of data points available that were used for analysis (88.9%) was similar for all 3 treatments.

Median preprandial abomasal pH was similar for all groups (Table 2), and luminal pH increased rapidly after suckling the test solutions at 0 and 12 h (Figure 2). Compared with suckling cow's milk alone, mean maximum postsuckling pH was increased by approximately 0.8 and 0.3 pH units when calves suckled the BACG-ORT or FA-ORT solution in cow's milk, respectively. Minimum and median postsuckling pH values were similar for all 3 treatments. The pH return time was longer when calves suckled the BACG-ORT or FA-ORT solution in cow's milk than when they suckled cow's milk alone.

Abomasal Emptying Rate

Mean values for actual C_{max} for plasma acetaminophen concentration and the pharmacokinetically calculated mean values for C_{max} were similar for all 3 treatments (Figure 3 and Table 2). Mean values for actual T_{max} for the BACG-ORT solution in cow's milk was longer than for cow's milk alone. The pharmacokinetically calculated mean values for T_{max} of the BACG-ORT and FA-ORT solutions in cow's milk were longer than for cow's milk alone.

The maximal plasma glucose concentration and area under the plasma glucose concentration – time curve for the first 6 h after suckling were greater when calves suckled the BACG-ORT solution in cow's milk than when they suckled cow's milk alone (Figure 4 and Table 2). In contrast, the area under the plasma glucose concentration – time curve for the first 6 h after suckling when calves suckled the FA-ORT solution in cow's milk was similar to that of cow's milk alone. The time to maximal plasma glucose concentration was similar for all 3 treatments.

Plasma Biochemical Analysis

The 3 test solutions produced similar decreases in plasma total protein concentration and small increases

Figure 2. Abomasal luminal pH-time relationship for 6 Holstein-Friesian calves suckling 2 L of cow's milk (○); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in cow's milk (group BACG, ●); or a formate- and acetate-containing ORT solution in cow's milk (group FA, ▲) at time 0 and 12 h. Data are expressed as mean $\pm$ SD.

in plasma volume (Figure 5). Addition of the BACG-ORT or FA-ORT solution resulted in mild systemic alkalinization, based on a significant main effect of treatment for total carbon dioxide. There were no differences in alkalinizing ability between the BACG-ORT and FA-ORT solution in cow's milk.

Abomasal Fluid Analysis

The measured osmolality of cow's milk was 292 ± 2 mOsm/kg. Addition of the BACG-ORT or FA-ORT solution to cow's milk caused a marked increase in solution osmolality (Figure 6 and Table 1). Abomasal fluid osmolality tended to increase shortly after suckling cow's milk, but the increase was not statistically significant. Abomasal fluid osmolality decreased after suckling the BACG-ORT or FA-ORT solutions in cow's milk, with abomasal fluid osmolality being similar at 6 h after suckling.

The measured total carbon dioxide concentration in BACG-ORT solutions in cow's milk was 8.9 ± 3.9 mmol/L. The total carbon dioxide concentrations in FA-ORT solutions in cow's milk and cow's milk alone were below the limit of quantification (<1.5 mmol/L). The measured SID of the abomasal fluid decreased

over time (Figure 6); however, both the BACG-ORT solution and the FA-ORT solution in cow's milk had a consistently greater measured SID than did cow's milk alone.

The sodium concentration in cow's milk was 16.2 ± 1.1 mmol/L. Addition of the BACG-ORT or FA-ORT solution to cow's milk markedly increased the solution sodium concentration (Figure 7 and Table 1). Abomasal fluid sodium concentration remained constant in calves suckling cow's milk, whereas the sodium concentration gradually declined in calves suckling the BACG-ORT or FA-ORT solution in cow's milk.

The potassium concentration in cow's milk was 43.6 ± 0.5 mmol/L. Addition of the BACG-ORT or FA-ORT solution to cow's milk slightly increased the solution potassium concentration (Figure 7 and Table 1). Abomasal fluid potassium concentration gradually declined in all calves after suckling the test solution.

The calcium concentration in cow's milk was 31.4 ± 0.9 mmol/L. Addition of the BACG-ORT solution to cow's milk slightly decreased the solution calcium concentration (Figure 7 and Table 1), whereas addition of the FA-ORT solution to cow's milk did not alter the solution calcium concentration. The abomasal fluid calcium concentration at time = 30 min was ap-

Figure 3. Plasma acetaminophen concentration-time relationship for 6 Holstein-Friesian calves suckling 2 L of cow's milk (○); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in cow's milk (group BACG, ●); or a formate- and acetate-containing ORT solution in cow's milk (group FA, ▲) at time 0 h. The 3 test solutions contained acetaminophen (50 mg/kg of BW). Data are expressed as mean $\pm$ SD.

proximately one-half that of the suckled solution, and remained constant for 6 h. The calcium concentration in abomasal fluid after suckling was similar for all 3 test solutions.

The chloride concentration in cow's milk was 25.0 ± 1.6 mmol/L. Addition of the BACG-ORT or FA-ORT solution to cow's milk markedly increased the solution chloride concentration (Figure 7 and Table 1). Abomasal fluid chloride concentration increased in an approximately linear fashion in calves suckling cow's milk, whereas the chloride concentration increased slightly when calves suckled the BACG-ORT or FA-ORT solution in cow's milk.

DISCUSSION

The main findings of the study reported here were that the addition of an ORT solution containing a low bicarbonate concentration (25 mmol/L), acetate (12 mmol/L), citrate (12 mmol/L), and glycine (7 mmol/L) to cow's milk increased in vitro clotting time

by approximately 6 min. However, a BACG-ORT solution in cow's milk clotted in vivo within 15 min after the beginning of suckling. Addition of the BACG-ORT solution to cow's milk increased the pH of cow's milk and abomasal fluid by approximately 0.3 pH units (in vitro) or 0.8 pH units (in vivo), increased solution osmolality, and slowed the rate of abomasal emptying, but did not change abomasal fluid calcium concentration. Our findings are in contrast to widely held beliefs that the addition of bicarbonate or citrate to cow's milk prevents clotting in the calf's abomasum (Heath et al., 1989; Naylor, 1992). However, our findings are consistent with those of other studies that indicate milk pH is the most important determinant of clotting time (White and Davies, 1958). In particular, as the pH of cow's milk increases from 6.4 to 7.2, there is a curvilinear increase in clotting time from 1.5 to 13 min (White and Davies, 1958). It is likely that in vitro studies documenting bicarbonate-containing ORT solutions delay or inhibit milk clotting when pH >6.6 (Naylor, 1992; Nappert and Spennick, 2003) are not relevant

Figure 4. Plasma glucose concentration-time relationship for 6 Holstein-Friesian calves suckling 2 L of cow's milk (○); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in cow's milk (group BACG, ●); or a formate- and acetate-containing ORT solution in cow's milk (group FA, ▲) at time 0 h. Data are expressed as mean $\pm$ SD * P < 0.05 compared with cow's milk at the same time. † P < 0.05 compared with the time = 0 min value.

to in vivo clotting. This is because the abomasal fluid pH after suckling is markedly less than the pH of the ORT solution as fed (Table 2 and Figure 2). However, because many commercially available ORT solutions in North America have a bicarbonate concentration of 40 to 80 mmol/L, it remains to be determined whether ORT solutions containing a greater bicarbonate concentration than 25 mmol/L delay or inhibit in vivo clotting. Preliminary results suggest that suckling ORT solutions containing >80 mmol/L of bicarbonate does not interfere with clotting of milk in the abomasum of the calf (Bachmann et al., 2007).

This appears to be the first in vivo study to evaluate the effect of solution formulation on clotting in the calf's abomasum directly, although a recent study indirectly identified the presence of clots in the abomasum of the calf by using transcutaneous ultrasonography (Miyazaki et al., 2007). A remarkable feature in the suckling calf is that the high CN concentration in cow's milk causes milk to clot within 10 min of entering the abomasum (Mortenson et al., 1935). Cow's milk clots when chymosin (rennin) interacts with CN and collo-

idal calcium phosphate (CCP) to form a curd, trapping CN and fat globules within the coagulum. The fluid whey, which contains carbohydrates and electrolytes, is released from the clot and passes quickly into the duodenum, whereas digestion of the milk clot occurs more slowly. It is widely believed that the calf requires normal curd formation in the abomasum for optimal growth and health, although direct evidence is lacking (Longenbach and Heinrichs, 1998).

The normal in vitro clotting time of bulk tank cow's milk is approximately 4 min (White and Davies, 1958), which is similar to the median clotting time for cow's milk in the study reported here. The clotting time of cow's milk after the addition of chymosin is dependent primarily on the pH (Foltman, 1970), particularly when pH > 6.6, and to a lesser extent on the temperature, the interaction between CN and CCP within the micelle, and the calcium ion activity within a micelle (White and Davies, 1958). Although it is widely believed that ORT solutions containing a high bicarbonate concentration inhibit clot formation in vivo, clot formation is inhibited only when the addition of bicarbonate

Figure 5. Plasma total carbon dioxide, total protein concentration, and percentage change in plasma volume for 6 Holstein-Friesian calves suckling 2 L of cow's milk (○); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in cow's milk (group BACG, ●); or a formate- and acetate-containing ORT solution in cow's milk (group FA, ▲) at time 0 h. Data are expressed as mean $\pm$ SD. † $P < 0.05$ compared with the time = 0 min value.

Figure 6. Osmolality and measured strong-ion difference for the 3 test solutions and abomasal fluid for 6 Holstein-Friesian calves suckling 2 L of cow's milk (○); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in cow's milk (group BACG, ●); or a formate- and acetate-containing ORT solution in cow's milk (group FA, ▲) at time 0 min. Data are expressed as mean $\pm$ SD. * $P < 0.05$ compared with cow's milk at the same time.

increases pH above 6.6, and particularly above 7.0 (Bywater, 1980). Moreover, the nature of the relationship between inhibition of clot formation and bicarbonate concentration has not been characterized. A decrease in milk pH below the normal value of 6.5 to 6.6 leads to a shorter clotting time because of increased chymosin activity and decreased electrostatic repulsion between micelles (Emmons et al., 1976). Increasing temperature leads to a shorter clotting time because of increased chymosin activity. Colloidal calcium phosphate is an insoluble calcium-phosphate complex $[\text{Ca}_3(\text{PO}_4)_2]$ that forms in cow's milk whenever fluid pH > 5.6 and is absent whenever fluid pH < 5.1 (Lucey et al., 1993). A

portion of the calcium present in cow's milk is therefore complexed with phosphate, and the presence of this pH-dependent CCP plays an important role in modulating the interactions between calcium and the charged groups on CN, particularly free carboxylic groups of aspartic and glutamic acids on β -LG and α -LA (Lucey, 2002).

When the minimum requirements for temperature, pH, CN concentration, and CCP have been met, the clotting time of cow's milk is dependent on the ionized calcium concentration (Udabage et al., 2001). The addition of citrate to cow's milk can therefore theoretically inhibit clotting because citrate is an anion that can form a complex with calcium ions. The addition of 14 or 15 mM citrate to a skim milk formulation at

pH 6.1 prevented clotting, whereas clotting occurred in the presence of a decreased citrate concentration (5.7 mM; Emmons et al., 1976; Jenkins and Emmons, 1983). However, it should be noted that the inhibition of clotting caused by citrate is pH dependent, with citrate failing to inhibit clotting at pH 5.5 (Jenkins and Emmons, 1982, 1983). The presence of 12 mM citrate in the BACG-ORT solution is therefore unlikely to produce a physiologically important *in vivo* effect on milk clotting, as shown in the study reported here. Moreover, although highly ionized calcium concentrations *in vitro* are invariably associated with short clotting times, ionized calcium concentrations below 3 mmol/L (12 mg/dL) have no relationship to the *in vitro* clotting time of cow's milk (White and Davies, 1958).

Figure 7. Sodium, potassium, calcium, and chloride concentrations for the 3 test solutions and abomasal fluid for 6 Holstein-Friesian calves suckling 2 L of cow's milk (○); a bicarbonate-, acetate-, citrate-, and glycine-containing oral rehydration therapy (ORT) solution in cow's milk (group BACG, ●); or a formate- and acetate-containing ORT solution in cow's milk (group FA, ▲) at time 0 min. Data are expressed as mean $\pm$ SD. * $P < 0.05$ compared with cow's milk at the same time.

The change in abomasal fluid pH in calves of 50 kg of BW after suckling 2 L of cow's milk (equivalent to 5% of BW) in the study reported here was characterized by a maximal *in vivo* pH of 5.42 and a pH return time of 144 min. For comparison, the change in abomasal fluid pH in calves of 46 kg of BW after suckling 2.76 L of cow's milk (equivalent to 6% of BW) was characterized by a maximal *in vivo* pH of 6.07 and a pH return time of 320 min (Constable et al., 2005a). Similarly, the change in abomasal fluid pH in calves of 54 kg of BW after suckling 2.16 L of cow's milk (equivalent to 4% of BW) was characterized by a maximal *in vivo* pH of 4.75 and a pH return time of 210 min (Reinhold et al., 2006). The volume suckled therefore influences the luminal pH-time relationship and maximal *in vivo* pH in the 12-h period of suckling. Prolongation of the *in vivo* clotting time is therefore more likely to occur when larger volumes of an ORT solution in cow's milk are fed, because luminal pH will initially be greater and remain greater for a longer period of time.

Cow's milk is buffered by soluble phosphate, CCP, citrate, bicarbonate, CN, and whey proteins (Salaün et al., 2005). Addition of the BACG-ORT solution to cow's milk added 3 quantitatively important buffers (bicarbonate, acetate, and citrate), whereas addition of the FA-ORT-solution to cow's milk added 2 quantitatively important buffers (formate and acetate). The acid titration curves (Figure 1) differed for the 3 test solutions because the buffers differed in the 3 test solutions. Acid titration of cow's milk typically reveals maximum buffering at approximately pH 5.1 (Lucey et al., 1993), as observed in the study reported here. This buffering peak is due to phosphate; this is because acidification of cow's milk below pH 5.6 causes the insoluble CCP to dissolve, thereby liberating phosphate for buffering (Lucey et al., 1993).

Typical values for β of bovine milk range from 0.018 to 0.040 (mol/L)/pH unit (Wiley, 1935; Lucey et al., 1993), similar to that found in the study reported here (Figure 1). The buffer value of the BACG-ORT solution in cow's milk was, in general, greater than that of cow's milk over the acid titration of pH range 2.0 to 6.5. This result was due in part to the presence of citrate in the BACG-ORT solution. Citric acid has 3 dissociation constants ($pK_1 = 3.1$, $pK_2 = 4.4$, $pK_3 = 5.5$) that are so close that the buffering effect of the citric acid-sodium citrate solution is approximately constant between pH 2.5 and 6.0. The BACG-ORT solution in cow's milk had a peak buffer value at approximately 4.85, which corresponded to the pK_a of acetic acid. The buffer value of the FA-ORT solution in cow's milk was, in general, greater than that of cow's milk over the titration range studied, and was markedly greater over the pH range of 2.8 to 4.2 because of the presence of formic acid, which

has an approximate pK_a value of 3.8. The FA-ORT solution in cow's milk also had a peak buffer value at approximately 4.85, similar to the BACG-ORT solution, because of the presence of acetate.

There are 3 quantitatively important components in the BACG-ORT solution in cow's milk that complex calcium: phosphate, citrate, and bicarbonate. Typical milk concentrations of total calcium and phosphate in cow's milk are 31 mmol/L (125 mg/dL) and 37 mmol/L (114 mg/dL), respectively (Cerbulis and Farrell, 1976). The typical milk concentration of citric acid in cow's milk is 9.0 mmol/L (172 mg/dL; Reinart and Nesbitt, 1959). Because cow's milk contains quantitatively important concentrations of phosphate and citrate, a portion of the total calcium concentration in milk is always in a complexed form, particularly at normal pH values for milk (6.5 to 6.6) and *in vivo* when abomasal fluid pH > 5.6. Formation of calcium complexes explains why addition of the BACG-ORT solution to cow's milk decreased the measured calcium concentration (Table 1), because bicarbonate and citrate can complex calcium. Although the maximal postsuckling abomasal fluid pH was 6.2, inspection of the luminal pH-time curve (Figure 2) indicated that abomasal fluid pH after suckling the BACG-ORT solution in cow's milk was < 5.6 for the vast majority of the time. This means that the formation of calcium complexes *in vivo* plays no or a minimal role in the observed decrease in abomasal fluid calcium concentration from 30 min onward after suckling (Figure 7). Instead, it is likely that the postsuckling decrease in calcium concentration is associated with the clotting of milk, whereby calcium is bound to CN and is not measurable in the whey portion.

Addition of the BACG-ORT solution to cow's milk slowed the rate of abomasal emptying, as assessed by pH return time (Table 2 and Figure 2) and acetaminophen absorption (Figure 3). This effect was most likely due to the increased osmolality and high glucose content. An abomasal fluid osmolality of 600 mOsm/kg markedly slows the rate of abomasal emptying (Sen et al., 2006). Increased abomasal fluid and plasma glucose concentrations also slow the rate of abomasal emptying by activating duodenal receptors and reflexively decreasing abomasal motility, and probably by glucose-induced hyperinsulinemia (Sen et al., 2006). The presence of acetate and citrate in the BACG-ORT solution was unlikely to have contributed to the slowed abomasal emptying rate because the presence of up to 80 mmol/L of acetate and 44 mmol/L of citrate in an ingested solution does not alter the gastric emptying rate in humans (Hunt and Knox, 1968), and because 150 mmol/L of sodium acetate did not alter the emptying rate in suckling calves (Marshall et al., 2008). The slower rate of emptying in the BACG-ORT solution

could be viewed as being beneficial in that the net effect was a sustained fluid, electrolyte, and nutrient delivery to the small intestine. It should be noted that addition of the BACG-ORT solution to cow's milk resulted in a more than 4-fold increase in solution sodium concentration (Table 1). Because the rate of rehydration is driven by the rate at which sodium and agents that facilitate sodium absorption (glucose, acetate, citrate, glycine) are presented to the small intestine, a small reduction in the rate of abomasal emptying in the presence of a marked increase in solution sodium concentration will result in a more rapid rate of delivery of sodium to the small intestine per unit time.

Cow's milk is isotonic to plasma. We observed that abomasal fluid osmolality tended to increase, but not significantly, over the 6-h period after calves suckled cow's milk. This finding is similar to that reported in 1964 (Ash, 1964), and the small but nonsignificant increase in osmolality was most likely due to the breakdown of milk protein during digestion. Hyperosmolar solutions have the theoretical disadvantage that they may create a transient dehydration after feeding because the hypertonicity of abomasal fluid contents causes movement of water from the extracellular space into the abomasum. Administration of a hypertonic ORT solution (698 mOsm/kg) at 37 mL/kg produced a transient dehydration for <30 min in calves with dehydration and diarrhea (Jones et al., 1984), but not in euvoletic calves (Levy et al., 1990). We administered a hypertonic ORT solution (619 mOsm/kg) at approximately 40 mL/kg and did not see a transient dehydration. Taken together, these findings indicate that transient dehydration attributable to suckling a hypertonic ORT solution, if present, is likely to be clinically unimportant.

Glucose in the BACG-ORT solution in cow's milk was fed at approximately 1.2 g/kg of BW (glucose) or 2.3 g/kg of BW (glucose in the BACG-ORT solution and glucose derived from lactose, assuming complete availability) every 12 h. Although plasma glucose concentration exceeded the renal threshold in the euvoletic calves in this study, we believe it is unlikely that the same magnitude of hyperglycemia would be seen when treating calves with diarrhea. This is because diarrheic calves are usually hypoglycemic (Jones et al., 1984), and treatment of diarrheic calves with an ORT solution containing glucose at 2.5 g/kg of BW produced mean normal plasma glucose concentrations of 130 mg/dL (Jones et al., 1984), below the renal threshold for glucose (Nouri and Constable, 2006).

Systemic alkalization is an important treatment goal when administering an ORT solution to calves with diarrhea (Naylor et al., 1990; Roussel and Kasari, 1990; Constable et al., 2001, 2005a,b; Nouri and Con-

stable, 2006). Both the BACG-ORT and FA-ORT solutions examined in this study were alkalizing relative to cow's milk alone, as indicated by a significant effect of treatment on total carbon dioxide concentration in serum (Figure 5). Systemic alkalization occurred as a result of an effective SID of 88 mEq/L for the BACG-ORT solution and 124 mEq/L for the FA-ORT solution (Table 1), and an associated increase in measured SID of abomasal fluid relative to that of cow's milk alone (Figure 6). Three alkalizing agents (bicarbonate, acetate, citrate) were included in the BACG-ORT solution to provide rapid systemic alkalization after absorption of bicarbonate and to provide a more sustained alkalizing effect after absorption and metabolism of acetate and citrate. Bicarbonate is alkalizing because it accepts a proton, as indicated by the Henderson-Hasselbalch equation, whereby

The alkalizing reactions for acetate (CH_3COO^-) and dihydrogen citrate [$\text{CH}_2\text{-COH}\cdot\text{CH}_2(\text{COOH})_2(\text{COO}^-)$] are as follows:

with bicarbonate generation resulting in the buffering of free protons. The SID theory offers an attractive alternative explanation for the buffering effect of sodium bicarbonate, sodium acetate, and monosodium citrate in that administration of these 3 salts increases abomasal fluid SID (Figure 6) and ultimately increases the plasma SID. This is because the strong cation (sodium) remains after bicarbonate is eliminated via respiration or the strong anion (acetate, dihydrogen citrate) is metabolized to bicarbonate and then eliminated (Constable, 1999). Assuming complete metabolism of acetate and dihydrogen citrate to bicarbonate in 2 L of BACG-ORT solution in cow's milk, a total of 98 mmol of bicarbonate would be produced: 50 mmol from bicarbonate, 24 mmol from acetate, and 24 mmol from citrate. The calves in the study reported here had an approximate BW of 50 kg, which is equivalent to an extracellular fluid volume of 30 L, assuming the extracellular fluid volume is 60% of the BW of a neonatal calf (Roussel and Kasari, 1990). Assuming instantaneous distribution, an amount of 98 mmol of bicarbonate in 30 L of extracellular fluid volume would be expected to increase plasma bicarbonate concentration by 3.3 mmol/L in a euhydrated calf, whereas the observed increase in total

carbon dioxide was approximately 2 mmol/L (Figure 5). The difference between the calculated and observed response was attributed to delayed or incomplete absorption and acid-base homeostatic mechanisms in the calf. In addition, metabolism of citrate may occur at a slower rate than acetate metabolism in calves, because intravenous administration of 68 to 80 mmol of trisodium citrate to 18-d-old calves did not alter blood pH, but induced clinical signs suggestive of hypocalcemia (Naylor and Forsyth, 1986). Orally administered citrate has been shown to be mildly alkalinizing in milk-fed calves (Den Hartog et al., 1989).

The FA-ORT solution in cow's milk has 2 alkalinizing agents, formate and acetate. Formate (formiate) is an anion (HCOO^-) derived from formic acid (HCOOH), with a pK_a of approximately 3.7 (Hanzlik et al., 2005). Formic acid is widely used as a preservative and antibacterial agent in animal feed. Because addition of the FA-ORT solution to cow's milk resulted in a decreased ORT solution pH, it is likely that formic acid, rather than sodium formate, was used to formulate the FA-ORT. Formate is metabolized to carbon dioxide and water (Hanzlik et al., 2005) and should therefore be alkalinizing once metabolized (Constable, 1999), as observed in this study. The FA-ORT solution would be expected to increase plasma bicarbonate concentration by 5.7 mmol/L (based on a calculation process similar to that for the BACG-ORT solution), whereas the observed increase in total carbon dioxide was approximately 2 mmol/L (Figure 5). It should be noted that the maximum theoretical alkalinizing ability for the BACG-ORT and FA-ORT solutions in cow's milk is an increase in plasma bicarbonate concentration of 6.6 and 11.4 mmol/L, respectively, assuming calves with diarrhea and strong-ion (metabolic) acidosis are fed 2 L of the solution twice daily. Typical biochemical changes in calves with diarrhea are a reduction in blood pH by 0.21 pH units and a decrease in plasma bicarbonate concentration of 9.7 mmol/L (Groutides and Michell, 1990). An initial fluid volume of 6 L is usually required to correct free-water deficits and ongoing fluid losses in calves with diarrhea. Two liters of the BACG-ORT solution would therefore need to be administered at least 3 times within the first 24 h of treatment to correct the typical bicarbonate deficit in a 50-kg calf with diarrhea.

The measured concentrations of electrolytes in cow's milk in this study were similar to those reported previously (Table 1), with the exception being a decreased sodium concentration. This result was most likely due to ionic binding of sodium to negative charges on CN in the milk clot, which would effectively decrease the sodium concentration in whey. For comparison, abomasal fluid concentrations in 16-d-old calves before and after

suckling cow's milk were sodium, 33 to 60 mmol/L; potassium, 10 to 30 mmol/L; and chlorine, 76 to 136 mmol/L (Mylrea, 1966). Abomasal fluid concentrations in 15-d-old calves after a 36- to 40-h fast were sodium, 69 mmol/L; potassium, 8 mmol/L; and chlorine, 131 mmol/L (Mylrea, 1968).

In conclusion, the results of the study reported here in healthy euvoletic calves indicated that the addition of a low-bicarbonate ORT solution to cow's milk did not affect milk clotting *in vivo*. Recommendations based on the results of *in vitro* studies that bicarbonate- or citrate-containing ORT solutions should not be fed concurrently with cow's milk do not appear to be relevant to *in vivo* conditions when 2 L of a low-bicarbonate (25 mmol/L), low-citrate (12 mmol/L) ORT solution is fed.

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