DAIRY TEAM
KANSAS STATE UNIVERSITY

DAIRY RESEARCH 2020

K-STATE
Research and Extension

KANSAS STATE UNIVERSITY AGRICULTURAL EXPERIMENT STATION AND COOPERATIVE EXTENSION SERVICE

DAIRY RESEARCH 2020

Contents

Foreword

Kansas State University is pleased to present the 2020 Dairy Research Report of Progress and proud to serve the growing Kansas dairy industry. Our state maintains strong dairy growth, posting a 3% increase in total milk production in 2019, the seventh highest annual growth rate in the nation. Over the last 10 years, milk production in Kansas has increased by 53%. At the end of 2019, 163,000 Kansas cows averaged 23,429 lb annually, ranking the state 16th in total milk production (3.8 billion lb). Kansas had 270 dairy operations and averages 604 cows per herd (12th in herd size), with 7 processing plants handling much of that volume.[1]

Selected production traits of our Kansas State University Dairy Teaching and Research Center (DTRC) herd are shown below. The excellent functioning of our herd is largely a tribute to the dedication of our staff: Michael Scheffel (manager), Robert Feist, Kris Frey, Ben Sims, Eulises Corrales, Alexandrea Eckert, Tony Hecht, Tyler Ohlde, and Bobby Ramming. Special thanks to the many graduate and undergraduate students for their technical assistance in our laboratories and at the DTRC. We also acknowledge the support and cooperation of the Heart of America Dairy Herd Improvement Association (DHIA) for its assistance in handling research milk samples.

Kansas State University Dairy Teaching and Research Center Herd[1]

Cows, total no.	259
Rolling herd milk, lb	31,537
Rolling herd fat, lb	1,090
Rolling herd protein, lb	922
Somatic cell count × 1,000	104
Calving interval, mo.	13.2

[1]December 8, 2020, test day (milking 2 to 3 times daily).

The sustained increases in productivity and efficiency on dairy farms in Kansas and across the U.S. are largely driven by improved technology and management decisions by dairy producers. It is our hope that the type of research presented in this report contributes to those improvements and helps to enhance the quality of dairy products to increase consumption. Thorough, quality research is not only time-intensive and meticulous, but also expensive. Nevertheless, studies have demonstrated that each dollar spent for research yields a 30 to 50% return in practical application. Those interested in supporting dairy research are encouraged to consider participation in the Livestock and Meat Industry Council (LMIC), a philanthropic organization dedicated to furthering academic and research pursuits by the Department of Animal Sciences and Industry. Additional details about the LMIC are found at the end of this report.

On behalf of the K-State Dairy Team
M.J. Brouk, Editor 2020 Dairy Research Report of Progress

[1] Progressive Dairyman, *https://www.progressivepublish.com/downloads/2020/general/2019-pd-stats-highres.pdf*.

Characterization of a Commercial Whey Protein Hydrolysate and Its Use as a Binding Agent in the Whey Protein Isolate Agglomeration Process

B. Zaitoun, N. Palmer,[1] and J.K. Amamcharla

Summary

Soy lecithin is a commonly used binder in agglomerating dairy powders. Due to the increase in consumer awareness on "clean label" and also to increase the shelf-life of agglomerated whey protein isolate (WPI), the demand of lecithin-free agglomerated WPI has increased. In this work, whey protein hydrolysate (WPH) was utilized as a binder to facilitate the agglomeration of WPI. The first objective was to characterize the chemical properties of three lots of WPH obtained from a commercial manufacturer. The degree of hydrolysis (DH) of WPH was 13.82–15.35% and not significantly ($P > 0.05$) different between the lots. It was observed from the high-performance liquid chromatography (HPLC) that the major whey proteins were completely hydrolyzed indicating a consistent hydrolysis between the lots. The second objective of the study was to evaluate the effectiveness of WPH as a binder in WPI wet agglomeration. After the agglomeration was performed, agglomerated WPI samples were stored at 25°C (77°F) and analyzed for moisture, water activity, relative dissolution index (RDI), and emulsifying capacity.

Moisture content (MC) of agglomerated samples was in the range of 3–15%, whereas water activity was within the range of 0.08–0.80. There was a significant ($P < 0.05$) difference in both moisture content and water activity among the treatments. Per-wet mass, flow rate, and the WPH concentration had significant ($P < 0.05$) effects on the MC. Moreover, all interactions among the main effects also had a significant ($P < 0.05$) effect on MC. High MC and water activity were observed for the treatments with higher pre-wet volume and higher flow rate and also resulted in clumping of the powders. The treatment that had 60 g of pre-wet, 20% WPH concentration, and 5.6 mL/min flow rate combination had the highest RDI among all the samples. In conclusion, WPH can be used as a potential alternative to soy lecithin in WPI wet agglomeration.

Introduction

Whey is a co-product obtained during the manufacturing of cheese. The major proteins in whey include β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulins (Ig). Whey proteins (WPs) are highly soluble and have unique physiochemical characteristics that influence their functionality in food applications such as gelation, emulsification, and foaming. This makes WP a beneficial ingredient in various food products. However, some approaches are performed to modify WPs' functional and physiochemical properties such as chemical, physical, and enzymatic treatments. The most recent research on protein modification includes Maillard conjugation; physical modification such as high-pressure treatment and heat-induced polymerization (thermal treatment); and enzymatic modifications. Enzymatic hydrolysis of WP is measured by the degree of hydrolysis, which is defined as the percentage of peptide bonds cleaved. A low DH (<10%) is sufficient for improving the physiochemical properties of WPs. Whereas a DH (>10%) is more suitable for improving the biological functions of the resultant peptides such as antimicrobial, antioxidant, antihypertensive, and immunomodulatory functions.

[1] Glanbia Nutritionals, Twin Falls, ID, 83301.

Enzymatic hydrolysis of WPs was extensively studied by the researchers. It was proven that hydrolysis improves WPs' digestibility and nutritional value and reduces allergenicity, which makes it a suitable ingredient in infant formula. Hydrolysis also improves the solubility and the emulsifying capacity at alkaline pH of the whey protein hydrolysate, which makes it a desirable ingredient in high-protein beverages and nutritional bars. Moreover, the hydrolysate can be used as an encapsulant for protection and delivery of bioactive components such as omega-3 oil and probiotic organisms. It was reported that the hydrolysate worked as an excellent binder in the WPI agglomeration process as it will be providing a "clean label" product, and it would increase the shelf-life of agglomerated whey protein isolate (WPI) as it makes it less susceptible to oxidation over the storage time.

Agglomeration improves the reconstitution properties of the powders due to the incorporation of air between powder particles, which makes the water penetration into these particles easier during subsequent rehydration. Therefore, the agglomerates readily disperse and dissolve quickly compared to non-agglomerated powders. In the present study, the first objective was to characterize the physical and chemical properties of three lots of a commercial WPH. Subsequently, optimizing and evaluating the effectiveness of WPH as a binder in WPI wet agglomeration was investigated as a second objective.

Experimental Procedures

Three lots of WPH and one lot of WPI were obtained from a commercial manufacturer (Glanbia Nutritionals, Twin Falls, ID). Initially, chemical and physical properties of WPH and WPI were analyzed in terms of peptide characterization, degree of hydrolysis, zeta potential, color, bulk, and tapped densities to evaluate the consistency of the enzymatic hydrolysis. After determining the similarities and differences among WPH lots, the effectiveness of using WPH as a binder in WPI wet agglomeration was evaluated. For this purpose, a 3 × 3 × 2 factorial design was conducted with pre-wet mass (60, 100, and 140 g), WPH concentration (15, 20, and 25%), and flow rate (4.0 and 5.6 mL/min) as independent variables. The other processing parameters such as the nozzle pressure, fluid bed pressure, and fluid bed temperature were set at 0.65 bar (9.43 psi), 0.45 bar (6.53 psi), and 60°C (140°F), respectively. WPI agglomeration was performed in a top-spray fluid bed granulator (Midi-Glatt, Binzen, Germany) as shown in Figure 2. All experiments were performed in triplicates using the three lots of WPH. Agglomeration was stopped when the temperature of the end powder reached 45°C (113°F). Agglomerated WPI samples were stored at 25°C (77°F) and analyzed for moisture, water activity, relative dissolution index (RDI), and emulsifying capacity.

Results and Discussion

The chemical compositions of WPI and WPH lots were provided by the manufacturer. Moisture content of the WPI sample was 3.61%, whereas WPH moisture content was within the range of 2.67–2.99%. The WPI protein content was 94.02% and WPH protein content was within the range of 91.57–92.28%. The major components of WPs (β-LG, α-LA, and BSA) were observed on the RP-HPLC chromatogram. In addition, two unidentified peaks were also observed. However, none of those peaks were detected on the WPH samples' chromatogram (Figure 1), which suggests the complete hydrolysis of these major whey proteins in the WPH lots.

The DH of WPH samples was 13.82–15.35% and it was not significantly ($P > 0.05$) different between the lots (Table 1). The mean particle size was within the range of 150.67–198.93 μm, and it was significantly different ($P < 0.05$) among the three WPH lots (Table 1). The mean particle size of WPH samples was bigger than the WPI due to the aggregation behavior of the hydrolysate. Zeta potential is an indicator of the surface charges of the particles and its stability to aggregation. The mean of zeta potential was within the range of -22.88 to -24.04 mV, and

there was no significant difference ($P > 0.05$) among the WPH lots (Table 1). Zeta potential of the hydrolysate samples was higher than that of the intact WPI. This is due to the increase of net charge on protein hydrolysate.

According to the American Dairy Products Institute (2002; Elmhurst, IL), MC should not exceed 6% by wt in dry whey products. MC for all treatments was in the normal range (<6%), except in treatments 14 (140 g, 15% and 5.6 mL/min), 16 (140 g, 20% and 5.6 mL/min) and 18 (140 g, 25% and 5.6 mL/min) that contained an MC of 14.79, 7.56, and 7.41%, respectively (Table 2). In these treatments, clumps were formed, therefore monitoring the temperature of the end product was difficult, which resulted in a large variation of MC in these treatments. The increase of pre-wet mass had highly influenced the MC (Figure 3). Flow rate had also increased the MC in agglomerated WPI, whereas, WPH concentration had a slight effect on the MC in agglomerated WPI. High pre-wet mass and high flow rate in these treatments caused a collapse in the fluid bed, which can be explained by the plasticization of the entire particle surface due to increased humidity of the air inside the fluid bed. Large clumps were formed and settled in the bottom of the fluid bed chamber (Figure 3), because the shear forces acting on the particles in the fluid bed were no longer sufficient to destroy the numerous sinter bridges generated, which led to a rapidly progressing cake formation. High MC in the powder may influence the shelf life due to Maillard reaction, creating the lumps and leading to microbial growth.

Dissolution characteristics of agglomerated WPI samples were evaluated using Focused Beam Reflectance Measurement (FBRM). The mean RDI of the agglomerated WPI samples manufactured as per the experimental design was 63.20–95.57% (Table 2). Treatment 4 (60 g, 20%, 5.6 mL/min) had the highest RDI of 95.57%, followed by treatment 6 (60 g, 25%, 5.6 mL/min) and treatment 5 (60 g, 25%, 4.0 mL/min) that had an RDI of 86.56 and 84.12%, respectively. There was no significant difference ($P > 0.05$) among these treatments. Treatment 3 (60 g, 20%, 4 mL/min) had the lowest RDI of 63.20 among the resultant agglomerated samples, followed by treatment 15 (140 g, 20%, 4 mL/min), and treatment 1 (60 g, 15%, 4 mL/min) 67.98 and 69.32 %, respectively. There was also no significant difference ($P > 0.05$) among those treatments. Pre-wet mass and flow rate had a significant effect ($P < 0.05$) on RDI. The WPH concentration did not have a significant ($P > 0.05$) difference as a main factor. However, its interactions with other factors were significantly different ($P < 0.05$). The interactions; pre-wet × WPH concentration, pre-wet × flow rate, WPH concentration × flow rate, and pre-wet × WPH concentration × flow rate were all significantly ($P < 0.05$) different.

Conclusions

Whey protein hydrolysate samples had similar chemical and physical properties indicating a consistent manufacturing process. Agglomeration conditions, especially the pre-wet mass and the flow rate, have affected primarily the moisture content, water activity, and the RDI of agglomerated samples. Some of the agglomerated samples have failed to meet the industrial and ADPI standards of dry whey products. The WPH concentration might have more impact on the physical properties of the agglomerates than it does on the functional properties.

Table 1. The degree of hydrolysis, mean particle size, and zeta potential of commercial whey protein hydrolysate (WPH) and whey protein isolate (WPI) samples in the present study

Samples	Degree of hydrolysis, %	Mean particle size (μm)	Zeta potential (mV)
WPH lot 1	14.80±0.35	181.23±0.60^{b}	-23.86±2.03
WPH lot 2	15.35±0.64	150.67±3.65^{c}	-24.04±1.40
WPH lot 3	13.82±0.39	198.93±9.77^{a}	-22.88±1.16
WPI	-	112.17±14.40	-19.59±1.58

$^{a-c}$ Means within a column with different superscripts are significantly different ($P > 0.05$).
All values are expressed as mean ± SD (n = 3).

Table 2. Moisture content (%), emulsifying capacity (g of oil/mg of protein), and relative dissolution index (%) of all agglomerated WPI treatments as per experimental design

Treatment	Pre-wet mass (g)	WPH concentration (%)	Flow rate (mL/min)	Moisture content (%)	Emulsifying capacity (g of oil/mg of protein)	Relative dissolution index (%)
1	60	15	4.0	3.36±0.15^{c}	4.66±0.29	69.324±3.79^{def}
2	60	15	5.6	5.37±1.60^{bc}	4.58±0.34	73.37±6.48^{bcdef}
3	60	20	4.0	3.35±0.52^{c}	4.59±0.29	63.20±0.94^{f}
4	60	20	5.6	4.15±0.16^{c}	4.40±0.42	95.57±4.32^{a}
5	60	25	4.0	3.48±0.52^{c}	4.40±0.52	84.12±5.02^{abc}
6	60	25	5.6	4.25±0.07^{c}	4.33±0.42	86.56±3.03^{ab}
7	100	15	4.0	4.48±0.73^{c}	4.93±0.20	81.83±3.24^{abcd}
8	100	15	5.6	5.39±0.37^{bc}	4.75±0.11	78.05±0.67^{bcdef}
9	100	20	4.0	4.26±0.41^{c}	4.80±0.13	82.83±5.19^{abcd}
10	100	20	5.6	3.77±0.25^{c}	4.82±0.20	75.25±5.55^{bcdef}
11	100	25	4.0	3.73±0.45^{c}	4.73±0.27	79.13±1.56^{bcde}
12	100	25	5.6	3.66±0.16^{c}	4.47±0.55	76.50±6.62^{bcdef}
13	140	15	4.0	3.30±0.13^{c}	4.63±0.11	74.19±4.91^{bcdef}
14	140	15	5.6	14.79±4.5^{a}	4.52±0.30	76.64±6.17^{bcdef}
15	140	20	4.0	3.51±0.06^{c}	4.75±0.29	67.98±4.58^{ef}
16	140	20	5.6	7.56±1.32^{b}	4.82±0.20	75.30±4.48^{bcdef}
17	140	25	4.0	3.56±0.1^{c}	4.76±0.25	70.64±4.18^{cdef}
18	140	25	5.6	7.41±3.46^{b}	4.71±0.39	75.37±10.42^{bcdef}

WPH = whey protein hydrolysate. WPI = whey protein isolate.
$^{a-f}$ Means within a column with different superscript are different ($P < 0.05$).
All values are expressed as mean ± SD (n = 3).

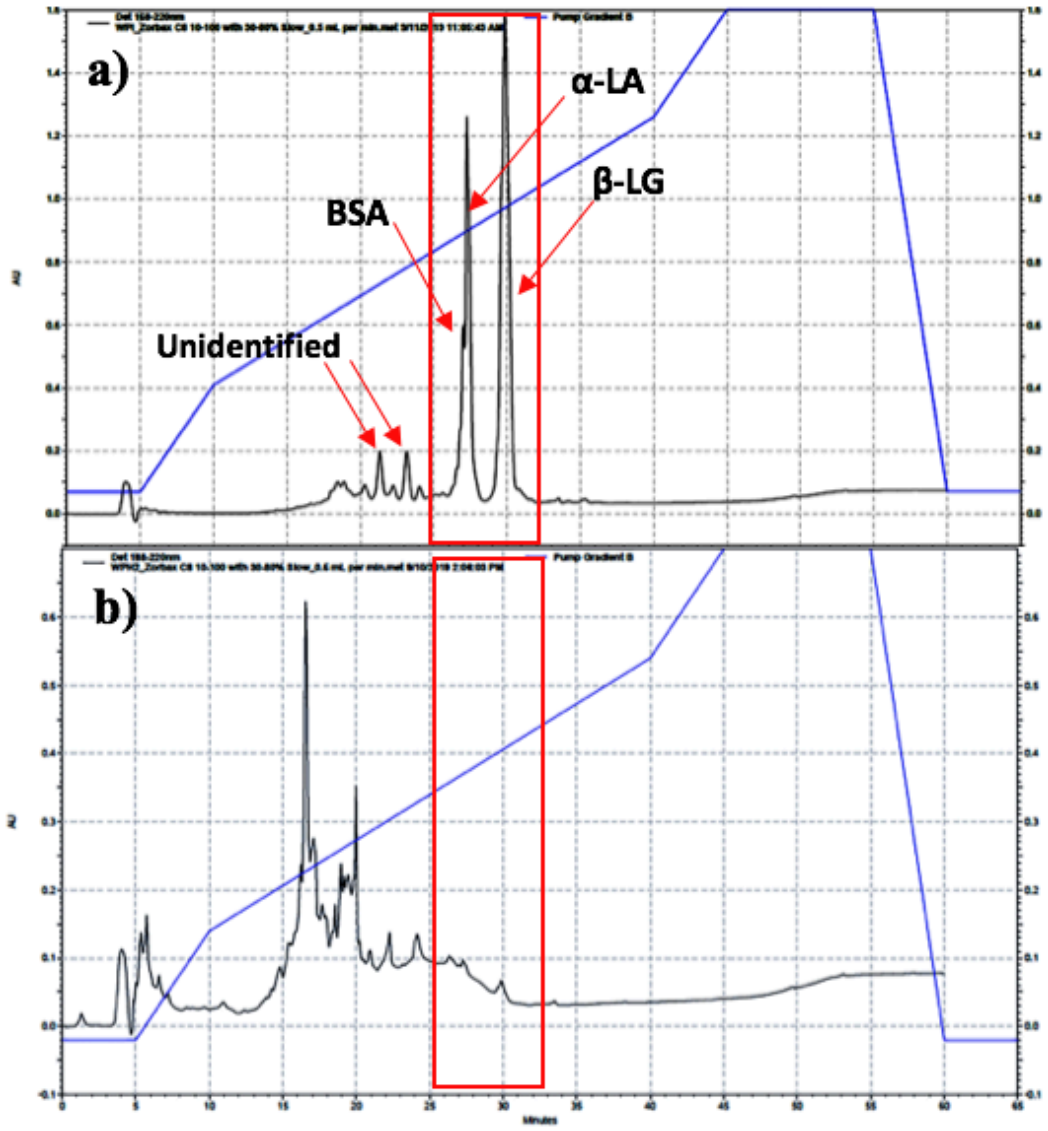

Figure 1. a) The Reversed-phase High-performance liquid chromatography (RP-HPLC) chromatogram of whey protein isolate, shows the major whey proteins before hydrolysis. b) The RP-HPLC chromatogram of whey protein hydrolysate (WPH). No peaks were observed in the times of 26.3, 27.2, and 29.9 min indicating a complete hydrolysis of the major whey proteins in WPH samples. β-LG = β-lactoglobulin. α-LA = α-lactalbumin. BSA = bovine serum albumin.

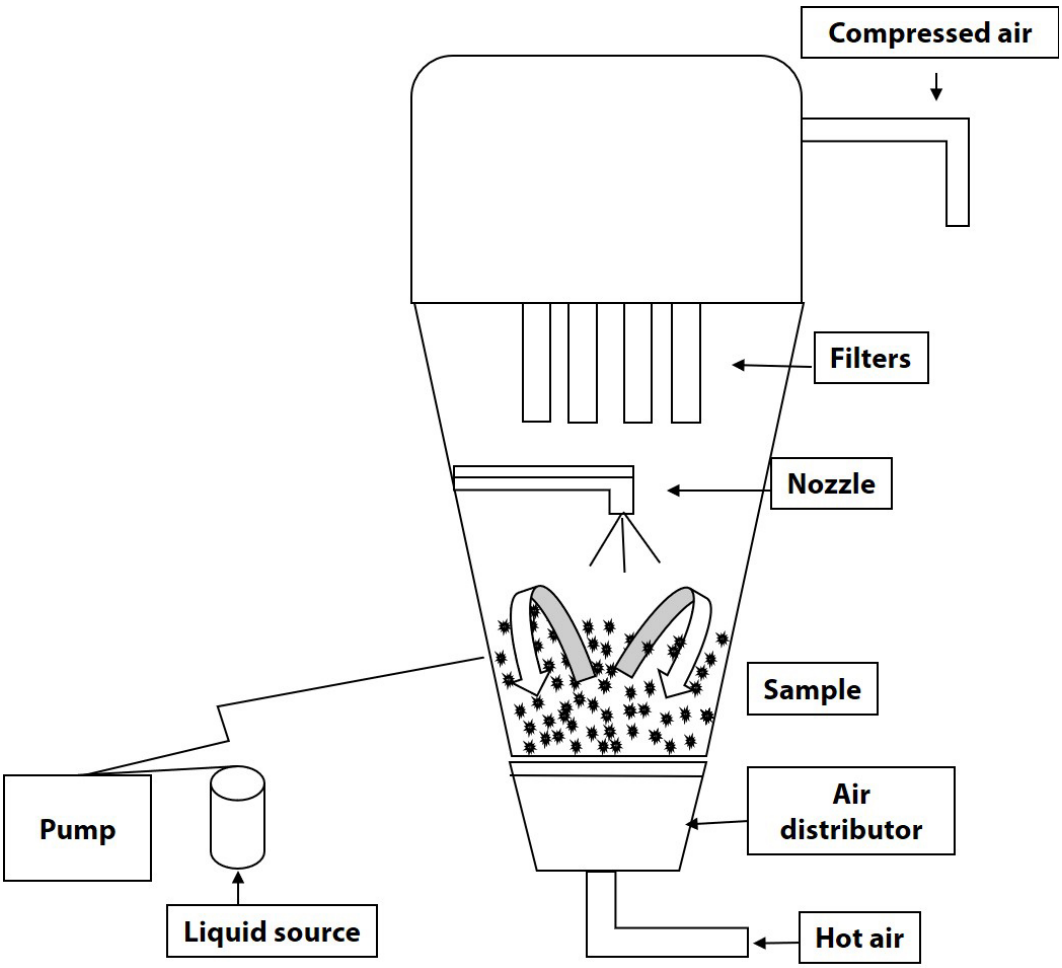

Figure 2. The top-spray fluid bed granulator (Midi-Glatt, Benzin, Germany) that was used in agglomerating whey protein isolate powder.

Figure 3. A representative image of agglomerated powder produced in treatment that had the combination of 140 g of pre-wet mass and 5.6 mL/min flow rate. The resultant powder did not meet the industrial specification of whey protein isolate.

Effect of Stirring and Static Heating on Fibrilization of Milk Whey Protein: A Processing Approach

G. Rathod and J. Amamcharla

Summary

When globular proteins such as whey proteins are converted to fibril structure, the functional properties, including viscosity and water holding capacity, are improved. Consequently, they can be used as functional ingredients in a variety of foods such as yogurt and bakery products. In this study, milk whey protein isolates were converted to fibrils by heating at low pH using two different approaches to reduce fibrilization time without compromising the quality of fibrils. Fibrils were made with an intervention of stirring to reduce fibrilization time. Continuous stirring improved the quality of fibrils and speed of the fibrilization.

Introduction

Food proteins are composed of amino acids (monomers) that link to form a peptide chain. The peptide chains develop various conformations, depending upon the makeup of amino acids, the charge groups, and the bonds between and within molecules. Most food proteins are found in the globular structure, and mostly found in tertiary and quaternary structures. Altering the native globular structure of a protein to a fibrillar structure can give new functional properties such as gelling, emulsification, and foaming capacity and surface activity. Structurally, fibrils are a few nanometers in diameter and a few microns in length and have a high aspect ratio, which can provide a space-filling ability to fibrils. These fibrils can give previously mentioned properties at a relatively lower protein content than the native proteins. The fibrilization of different plant-based food proteins such as soy protein, pea protein, maize protein, wheat protein, rice protein, and cottonseed protein have been extensively studied. Other than plant protein, animal proteins such as egg and milk proteins were also explored for fibril formation.

Milk proteins are composed of two principal proteins, casein and whey proteins, among which whey proteins, a globular protein, was extensively studied for fibril formation. There were several studies on the fibrilization of whey protein isolate (made from cheese whey) and a pure β-lactoglobulin fraction of whey proteins. Several researchers have tried different concentrations of whey protein (1–3%), with varying pH of 1.8 to 3 (below the isoelectric point of whey proteins) and different time-temperature combinations from 75–120°C (167-248°F) and 5–24 hours. Fibrils made by heating 2% whey proteins solution at pH 2 at 80°C (176°F) for 20 hours were reported best among all other combinations in terms of fibrils morphology, length, and maturity of fibrils. However, 20 hours is a very time-consuming process and required a lot of energy. A faster method is required to make the process feasible for industry. During fibril formation, proteins are hydrolyzed into smaller molecular protein fractions (2–8 kD) due to heat and acid. Within this hydrolysate, some proteins act as nuclei and other protein fractions arrange themselves in a systematic order to form fibrils. Hence, hydrolysis of protein and subsequent nuclei growth in terms of fibrils is most important to make fibrilization develop fast. Most of the reported study was done using static heating in a glass container where the movement of the peptides was lower, hence the growth of nuclei took time to form fibrils with maturity. The stirring of acidified protein solution can increase the overall rate of reaction and accelerate the fibril growth by enhancing the material flow in the solution. Stirring can be used as an intervention to reduce the fibrilization time of whey proteins. Therefore, the study was designed to see the effect of stirring over static heating in the time frame of 20 hours.

Experimental Procedures

Methodology

Milk whey protein isolate (mWPI) powders (4.3% moisture, 91.6% true protein (87.6% whey proteins and 12.4% casein), 1.4% lactose, and 2.1% ash) were rehydrated to 2% (w/w) solution on a protein basis with distilled water and stored overnight at 4°C (39.2°F) to ensure complete hydration. The pH of mWPI solution was adjusted to 2 using 6 N hydrochloric acid and stored overnight at 4°C (39.2°F). The pH-adjusted mWPI solutions were heated to 80°C (176°F) for 20 h using two different approaches. In approach 1, pH adjusted mWPI solution was filled in the 20 heat-resistant screw cap test tubes (8 mL, 17 mm dia. × 63 mm height) (DWK Life Science, Millville, NJ) and placed in water bath (Fisher Scientific, Waltham, MA) maintained at 80±0.5°C (176±0.9°F). Every hour one tube was taken out without disturbing other tubes and immediately cooled with iced water to 4°C (39.2°F) and labeled with the respective hour. In approach 2, mWPI solution was heated in a glass container using a temperature-controlled circulatory water bath (Cole Parmer, Vernon Hills, IL) under gentle continuous stirring using a magnetic stirrer. The glass container was sealed to ensure no moisture loss during heating. A thermometer was used to continuously monitor and record the temperature during the heating process. Every hour a 10 mL representative sample was drawn using a disposable pipette and transferred in a glass tube, with subsequent cooling and labeling similar to approach 1. Samples were stored in a refrigerator at 4°C (39.2°F) until further analysis. All the samples were analyzed for thioflavin T fluorescence value, transmission electron microscopy, gel electrophoresis, rheology, and particle size.

Confirmation of the Presence of mWPI Fibrils

Thioflavin T Fluorescence Value

Phosphate-sodium chloride buffer (10 mM phosphate (Fisher Scientific, Waltham, MA) and 150 mM NaCl (Fisher Scientific, Waltham, MA), pH 7.0) was prepared and stored overnight. Thioflavin T fluorescence stock solution (3 mM) was prepared by dissolving Thioflavin T reagent (Acros Organics, Fisher Scientific, Waltham, MA) in the phosphate-NaCl buffer. The Thioflavin T fluorescence stock solution was filtered through a 0.2 μm WhatmanTM syringe filter (GE Healthcare UK Limited, Buckinghamshire, UK) to remove any undissolved particles. The Thioflavin T fluorescence stock solution was stored at 4°C (39.2°F) in an amber-colored glass bottle covered with aluminum foil. The Thioflavin T fluorescence working solution was prepared by diluting the Thioflavin T fluorescence stock solution 50-fold in the phosphate-NaCl buffer (final Thioflavin T concentration 60 μM). For the assay, a 48 μL sample was added to 4 mL working solution; the mixture was vortexed using a mini vortex mixture (Fisher Scientific, Waltham, MA) and held at room temperature for 1 min before measuring in a spectrofluorometer (LS-55; Perkin Elmer, Waltham, MA). The excitation wavelength was fixed at 440 nm with a slit width of 10 nm and the emission wavelength was scanned from 460–500 nm with slit widths 5 nm. Fluorescence intensity recorded at 482 nm wavelength was taken as the Thioflavin T fluorescence value. The Thioflavin T fluorescence value of the Thioflavin T working solution was taken as blank and subtracted from all the measurements. For each sample, the triplicate analysis was carried out and the scans were averaged to improve the signal-to-noise ratio.

Transmission Electron Microscopy

Samples were prepared for transmission electron microscopy (TEM) by diluting the protein solutions to 0.05% w/w protein using deionized pH 2 water where pH of deionized water was adjusted with 6 N hydrochloric acid. Proteins (20 μL) were absorbed onto a Formvar/carbon-coated 200-mesh copper grids (Electron Microscopy Sciences, Fort Washington, PA) for 30–60 s. The excess solution was wicked off with filter paper, stained with 2% uranyl acetate aqueous solution (Ladd Research Industries, Inc., Burlington, VT) for 30–60 s and the excess stain was wicked off with filter paper. Grids were viewed by TEM at 100 kV (CM 100 TEM

(FEI Company, Hillsboro, OR)), and images were captured using a digital camera (Hamamatsu model C8484, Bridgewater, NJ) and AMT software (Advanced Microscopy Techniques, Chazy, NY). The length and width of the mWPI fibrils were measured and analyzed using ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD).

Tris Tricine Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Samples for gel electrophoresis were diluted to 0.5% protein content and vortexed. Samples were diluted with tris-tricine sample buffer in a 1:2 ratio and vortexed. They were incubated at 37°C (98.6°F) for 15 minutes. Then, a 15 µl sample was loaded in the 16.5% precast Tris-Tricine gel (BioRad Laboratories, Hercules, CA), fitted into electrophoresis assembly (BioRad Laboratories, Hercules, CA) filled with 1X Tris-Tricine sample buffer (BioRad, USA) prepared from 10X Tris-Tricine buffer by mixing sample buffer, and double-distilled water in 1:9 ratio. The gel was run on 20 mA for 15 minutes for stacking. Then, gel was adjusted to 25 mA for 90 minutes. The gel was fixed in 10% trichloroacetic acid solution for one hour. The gel was removed and dipped into a tray filled with staining solution (Coomassie brilliant blue R-250, BioRad, USA) with continuous shaking for 24 hours. After that, staining solution was replaced with a destaining solution (10% methanol, 10% acetic acid, 80% double distilled water) and kept for 24 hours with continuous shaking at room temperature. Images were taken with a camera and analyzed with ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD).

Rheology

Continuous rotational flow data were collected at 20°C (68°F) using a stress-strain-controlled rheometer (MCR-92 Anton Paar, Vernon Hills, IL) fitted with a 50 mm diameter stainless steel cone with angle 1° and 101 µm gap at a varied shear rate from 0.01 s^{-1} to 200 s^{-1}. The sample was transferred to the center of the base plate with a disposable pipette and maintained at 20°C (68°F). Then the cone and plate probe were lowered so that gap between the probe and base plate remained 101 µm. The excess sample was trimmed and observed for even spread between the probe and base plate before starting the analysis. Along with viscosity, the consistency coefficient and flow behavior index were also calculated using the power-law model.

$$\tau = K\dot{\gamma}^{\eta}$$

Where τ = shear stress, $\dot{\gamma}$ = shear strain, K = consistency coefficient, and η = flow behavior index.

Statistical Analysis

Statistical analysis was performed by repeated measure ANOVA by SAS v. 9.4 software (SAS Inst., Cary, NC).

Results and Discussion

Thioflavin T Fluorescence Value

Thioflavin T fluorescence value of the static heating and stirring are shown in Figure 1. Thioflavin T fluorescence value of unheated mWPI solution at pH 2 was 35.28±4.05 and 32.63±3.78 for static and stirring heating samples, respectively. The Thioflavin T value increased with an increase in time for both the samples. At 10 h, the Thioflavin T fluorescence value of static heating was 277.88±58.8 AU, which was significantly ($P < 0.05$) lower than that of stirring heating (417.65±48.09 AU). Further, at 20 h, the Thioflavin T fluorescence value of the static heating samples was 404.27±25.05 AU, which was significantly lower than stirring heating samples (683.07±90.62 AU) and even lower than the Thioflavin T fluorescence value of the stirring heating sample at 10 h (417.65±48.09 AU). Both stirring and static heating samples were significantly ($P < 0.05$) different during the whole 20 h period (Table 1).

The Thioflavin T fluorescence value is an indicator of fibrilization, which increases with an increase in fibrils formation. The result showed that stirring heating had a higher Thioflavin T fluorescence value than static heating during the whole 20-h duration, which indicated that stirring heating produced a higher number of fibrils than static heating. Further, fibril growth have been divided in three phases, an initial slow increase in the lag phase, the rapid increase in the log phase, and little rise in the stationary phase in previous studies. A similar pattern was seen for static heating where an increase in Thioflavin T fluorescence value was slow (lag phase) until 3 hours, after that it increased (log phase) faster until 10 hours, and then a slow increase (stationary phase) was seen until 20 h. However, in the case of stirring heating, Thioflavin T value was increased (log phase) throughout the 20 h period. Fibrils are formed by the rearrangement of low molecular weight peptides, which are produced from protein due to heat acid hydrolysis. In the static condition, the movement of these peptides was limited, hence the rate of fibril formation was slow. However, the formation of fibrils from the fibril nuclei will not be inhibited by the depletion of the peptide in the stirred solution, as would occur with static heating condition. Hence, stirring heating showed no stationary phase until 20 h.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) images are shown in Figure 2. The TEM images show fibril formation from the native globular protein to fibrillar form in both the methods starting from 2 h. In static heating, very low fibrils can be seen at 2 h, and with an increase in heating time fibrils formation can be seen but that is not as intensive as the stirring heating. Large aggregates and fibrillar aggregates can be seen with an increase in time. In contrast to that, stirring heating showed a uniform distributed network of fibrils, and the number of visible fibrils was higher as compared to static heating. Further, aggregates were also less in stirring heating as compared to static heating. At 14 h, fibril from stirring heating was clear, intact, and mature, which can be seen in the image at lower magnification (Figure 2A) and higher magnification (Figure 2B) with very little aggregation. Further heating leads to a decrease in visible fibrils (Figure 2A and 2B).

In the case of stirring heating, continuous movement of the solution facilitated uniform heating of mWPI solution, and thereby uniform protein hydrolysis in peptides. Further, during fibril formation, stirring of solution provided enough hydrolyzed protein to fibril nuclei to form fibrils. Hence, fibrils formed in stirring heating were uniform and identical, showing less fibrillar aggregation as compared to static heating. In static heating, fibrils were formed based on the availability of hydrolyzed protein from the surroundings of fibril nuclei in solution, which cannot be uniform due to lack of movement of the solution that was produced by the stirring heating, hence fibrils formed are not uniform. Further, in a static condition, fibrils had enough time to crosslink, which led to aggregation which can be seen in the TEM images (Figure 2).

Gel Electrophoresis

Tris Tricine SDS-PAGE images are shown in Figure 3. Initially, both static and stirring heating showed a clear band of different whey protein fractions, among which β-Lg and α-La are the major whey protein fractions, however, there was no whey protein less than 10 kD. Upon heating at pH 2, the concentration of intact whey proteins, α-lactalbumin, and β-lactoglobulin, was decreasing with an increase in heating time, and the concentration of small molecular weight fractions below 10 kD was increasing with heating time. As compared to stirring heating (Figure 3B), static heating (Figure 3A) showed a faster conversion of whey protein into small protein fractions (2–10 kD) from the 2 hours itself and showed less intact whey protein at the end of 20 h.

In the fibril formation process, proteins were hydrolyzed into smaller sized protein fractions of 2–8 kD and the fractions rearranged themselves into fibrils. Under the action of SDS, fibrils which are made from small-sized protein fractions are dissociated again and can be seen as

a separate gel band. Hence, the occurrence of a higher amount of small-sized protein could indicate a higher rate of hydrolysis which could enhance the formation of the fibril. But along with the number of small sized protein fractions, the systematic arrangement was needed for fibril formation. Stirring heating showed lower conversion of small-sized protein fractions, but stirring may have enhanced rearrangement of these small protein fractions and thereby fibril formation.

Rheology

Table 1 shows rheological data for the stirring and static heating samples from 0–20 h. In the stirring heating sample, viscosity (at $100S^{-1}$) increased from 1.08±0.03 to 1.37±0.19 mPa.S within 2 h, and increased continuously until it reached 6.08±0.09 mPaS at 19 h. Unlike this continuous increase, the static heating sample showed a definite increase in viscosity, but this rise was irregularly reached to 6.48±1.10 at 19 h. A similar increase can be seen in the result of the consistency coefficient where stirring heating showed a steady increase compared to the discontinuous increase in static heating. On a similar pattern, the flow behavior index also decreased with time in both the methods but stirring heating showed a gradual decrease compared to a discontinuous decrease in static heating. The probable reason could be the method of manufacture of the fibril. In stirring heating, pH-adjusted protein solution was continuously stirred, which facilitated even distribution of hydrolyzed protein fractions for fibrils formation, hence the fibrils formed were aligned in similar orientation and remained parallel to each other in the solution. So, when they were analyzed for rheology, they may have aligned parallel to the plane of the probe. They showed lower viscosity at static heating at a lower shear rate. At a higher shear rate both the methods showed almost similar viscosity. However, static heating fibrils may have formed a three-dimensional network during the fibrils formation process. These networks were broken due to shear stress, and fibrils aligned with the probe at higher shear. Therefore, at higher shear, not much difference in viscosity was seen and a similar phenomenon was replicated in consistency coefficient and flow behavior index.

Conclusions

Milk whey proteins are globular in structure in their native state. The proteins can be converted to fibrils to harness the functionality of fibrils such as gelling, emulsification, and foaming. Fibrils are produced by heating milk whey protein solution at low pH, which hydrolyzes the protein into small-sized protein fractions, and the fractions are arranged systematically into fibrils. In conventional heating, the time required to produce fibrils is long, and the quality of fibrils is compromised. To expedite this process and improve the quality of fibrils, stirring was introduced as a processing aid, which can increase the rate of fibril formation and can produce uniform fibrils. Thioflavin T fluorescence value and transmission electron microscopic images showed that good quality fibrils could be produced by stirring while heating. Time could be reduced to 14 h rather than 20 h heating in the conventional process. Rheological studies showed a consistent rise in viscosity in stirring heating over static heating. Hence, it could be said that stirring heating can produce good quality fibrils in less time.

Table 1. Rheology of the stirring and static heating samples

	Apparent viscosity (m.Pa.S) $100S^{-1}$		Consistency coefficient ($Pa.s^n$)		Flow behavior index	
Time, h	Stirring heating	Static heating	Stirring heating	Static heating	Stirring heating	Static heating
0	1.08±0.03 ^{A}	1.09±0.03 ^{A}	0.0011±0.0002 ^{A}	0.0012±0.0001 ^{A}	0.99±0.03 ^{A}	0.99±0.01 ^{A}
2*	1.37±0.19 ^{A}	1.16±0.03 ^{B}	0.0022±0.0007 ^{A}	0.0012±0.0001 ^{B}	0.91±0.04 ^{B}	0.99±0.01 ^{A}
3	1.89±0.05 ^{A}	1.34±0.07 ^{B}	0.0046±0.0002 ^{A}	0.0019±0.0005 ^{B}	0.81±0.01 ^{B}	0.94±0.05 ^{A}
4	2.23±0.19 ^{A}	1.75±0.55 ^{A}	0.0068±0.0009 ^{A}	0.0063±0.0057 ^{A}	0.76±0.02 ^{A}	0.79±0.14 ^{A}
5	2.77±0.31 ^{A}	3.58±1.23 ^{A}	0.0098±0.0012 ^{A}	0.0386±0.0364 ^{A}	0.72±0.01 ^{A}	0.55±0.14 ^{B}
6	3.29±0.24 ^{A}	4.01±1.55 ^{A}	0.0133±0.0013 ^{A}	0.0631±0.0628 ^{A}	0.7±0.01 ^{A}	0.49±0.16 ^{B}
7	3.68±0.21 ^{A}	4.79±1.53 ^{A}	0.0165±0.0022 ^{B}	0.0922±0.0738 ^{A}	0.68±0.02 ^{A}	0.42±0.12 ^{B}
8	4.19±0.39 ^{A}	4.21±0.35 ^{A}	0.0180±0.0021 ^{B}	0.0580±0.0164 ^{A}	0.68±0.02 ^{A}	0.44±0.05 ^{B}
9	4.44±0.29 ^{A}	4.20±0.56 ^{A}	0.0219±0.0015 ^{B}	0.0619±0.0219 ^{A}	0.66±0.01 ^{A}	0.43±0.05 ^{B}
10	4.72±0.13 ^{A}	2.96±1.01 ^{B}	0.0227±0.0014 ^{A}	0.0328±0.0294 ^{A}	0.66±0.02 ^{A}	0.54±0.12 ^{B}
11	4.92±0.16 ^{A}	5.22±1.56 ^{A}	0.0246±0.0009 ^{B}	0.1011±0.0644 ^{A}	0.65±0.02 ^{A}	0.4±0.09 ^{B}
12	5.20±0.14 ^{A}	3.69±1.21 ^{A}	0.0279±0.0029 ^{A}	0.0428±0.0331 ^{A}	0.64±0.02 ^{A}	0.51±0.1 ^{B}
13	5.27±0.16 ^{A}	5.63±1.63 ^{A}	0.0272±0.0011 ^{B}	0.1331±0.0490 ^{A}	0.65±0.02 ^{A}	0.33±0.03 ^{B}
14	5.31±0.07 ^{A}	5.01±0.91 ^{A}	0.0277±0.0010 ^{B}	0.1089±0.0320 ^{A}	0.64±0.01 ^{A}	0.35±0.06 ^{B}
15	5.49±0.06 ^{A}	5.64±2.58 ^{A}	0.0294±0.0020 ^{A}	1.5642±2.9061 ^{A}	0.64±0.02 ^{A}	0.41±0.17 ^{A}
16	5.06±0.90 ^{B}	5.62±1.10 ^{A}	0.0288±0.0068 ^{B}	0.1426±0.0234 ^{A}	0.63±0.02 ^{A}	0.3±0.07 ^{B}
17	6.09±0.14 ^{A}	3.84±0.83 ^{B}	0.0337±0.0038 ^{A}	0.0687±0.0513 ^{A}	0.63±0.02 ^{A}	0.41±0.11 ^{B}
18	6.07±0.11 ^{A}	6.48±1.23 ^{A}	0.0368±0.0006 ^{B}	0.2418±0.1185 ^{A}	0.61±0.01 ^{A}	0.23±0.08 ^{B}
19	6.08±0.09 ^{A}	6.48±1.10 ^{A}	0.0330±0.0023 ^{B}	0.1611±0.0485 ^{A}	0.63±0.02 ^{A}	0.31±0.04 ^{B}
20	5.94±0.13 ^{A}	6.06±1.01 ^{A}	0.0323±0.0040 ^{B}	0.1287±0.0446 ^{A}	0.64±0.02 ^{A}	0.35±0.06 ^{B}

$^{A-B}$ Mean within a column (static heating and stirring heating) with different superscripts are different ($P < 0.05$) n = 4.
*Data were not collected for hour 1.

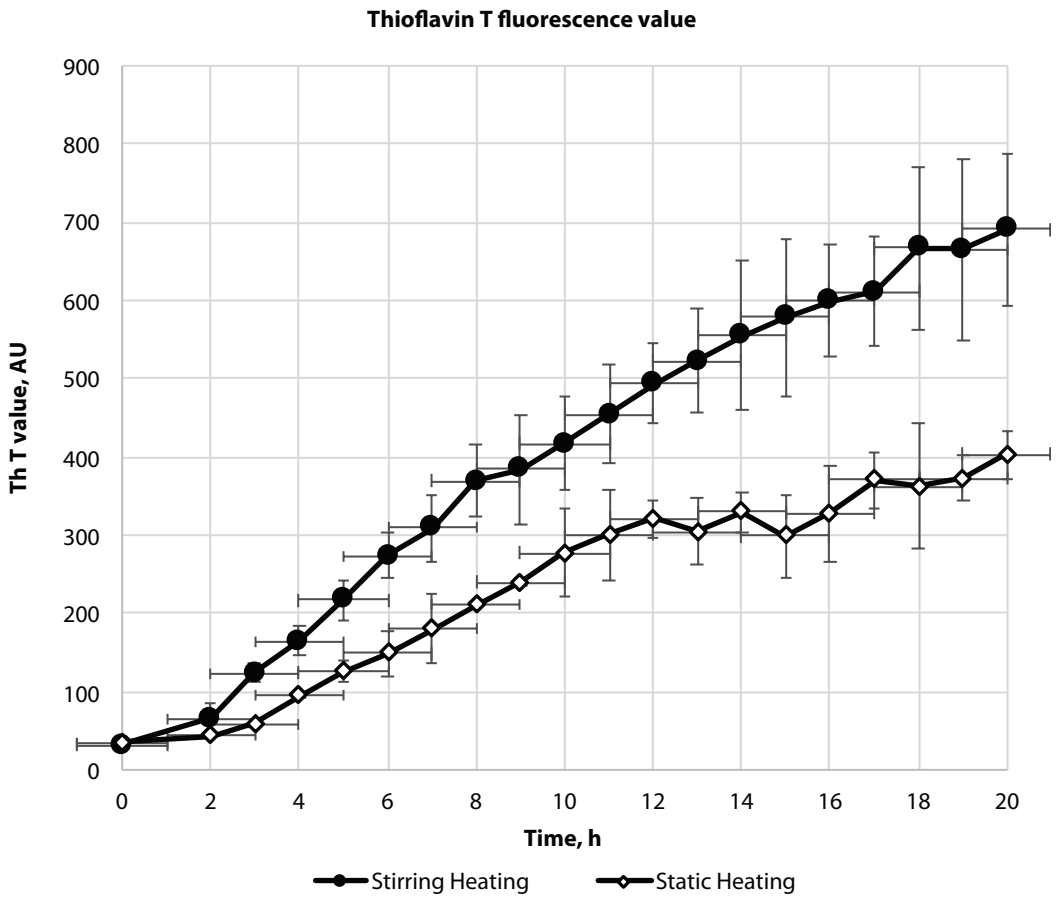

Figure 1. Thioflavin T value of stirring and static heating (0–20 h).

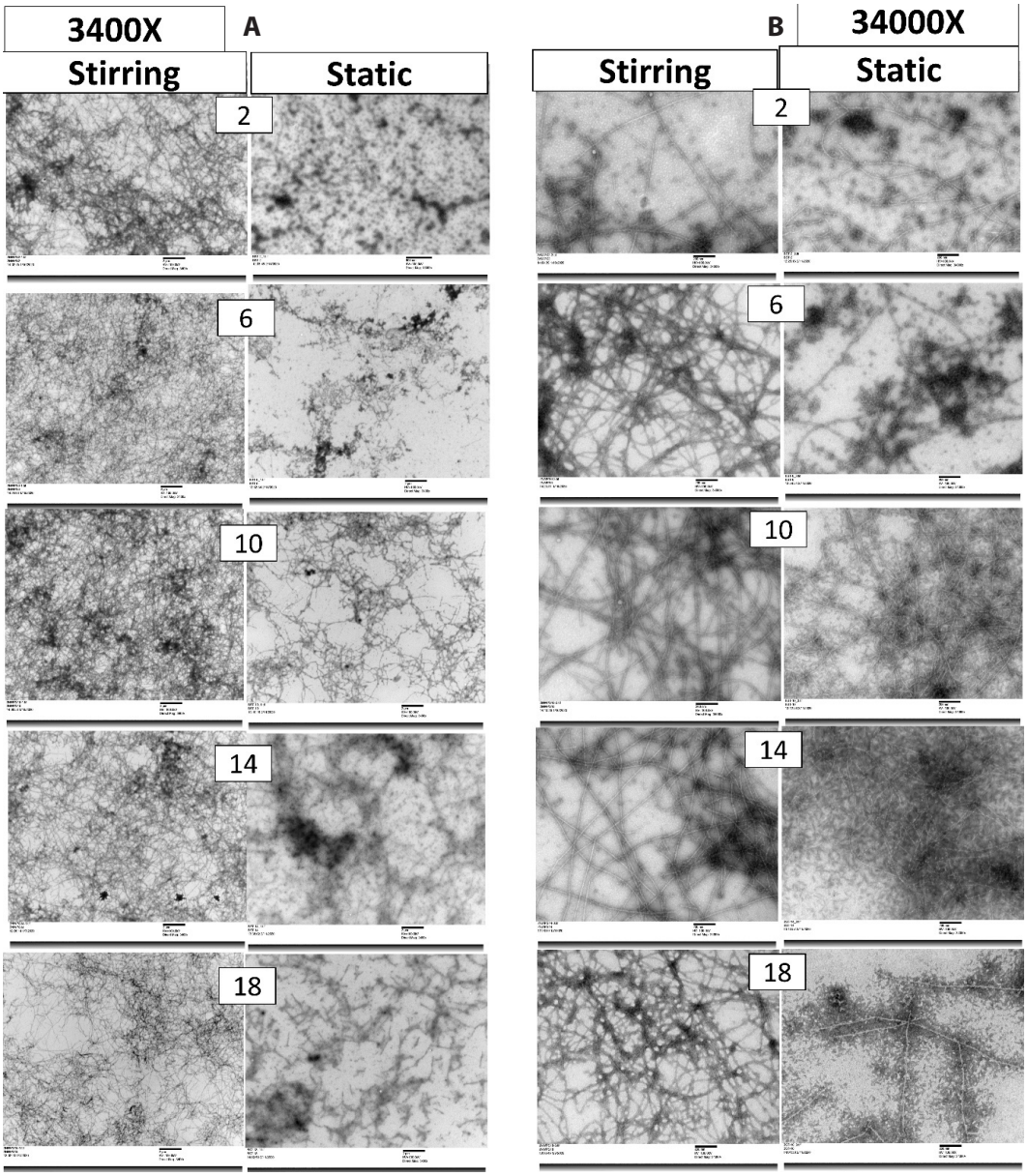

Figure 2. Transmission electron microscopy images of stirring and static heating samples at 80±0.5°C (176±0.9°F)at different hours. (A) Lower magnification. (B) Higher magnification.

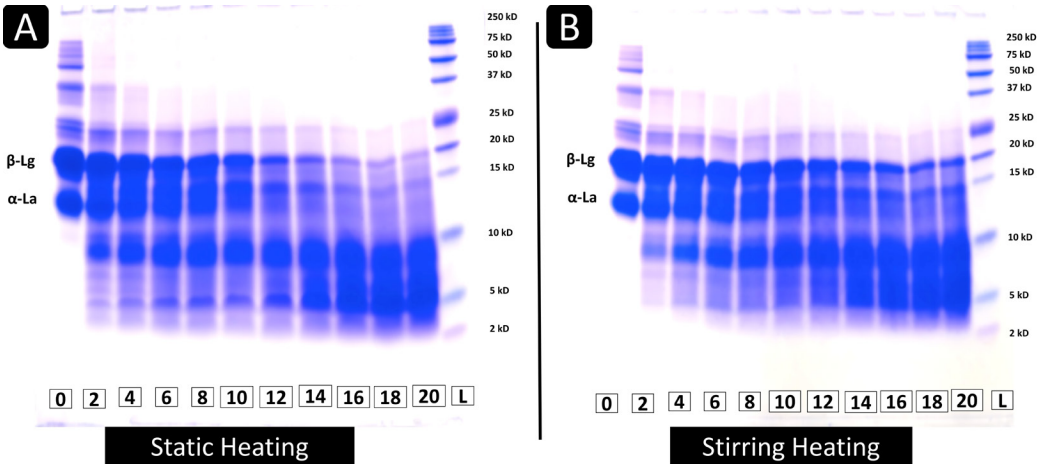

Figure 3. Tris-Tricine SDS PAGE of stirring heating sample (0–20 h, L-ladder). β-Lg = β-lactoglobulin. α-La = α-lactalbumin.

Complete Luteolysis and Pregnancy Per Insemination After Modifying the Standard 7-day Ovsynch Program in Dairy Cows

J.S. Stevenson and B. Atanasov

Summary

Two experiments were conducted with Holstein-Friesian cows in the Republic of North Macedonia and with Holstein cows in Kansas. We hypothesized that a single standard dose of $PGF_{2\alpha}$ injected on day 8 instead of day 7 in the standard Ovsynch program would increase the proportion of cows with complete luteolysis and increase the synchronization risk compared with control cows receiving a standard dose on day 7. Cows were treated with the standard program (Ov-7x1) or with Ov-8x1 experimental program in experiment 1, using only a single dose of $PGF_{2\alpha}$. In experiment 2, a third treatment was added (Ov-7x2), in which a second dose of $PGF_{2\alpha}$ was administered 24 hours after the first dose. Overall, the results in experiment 1 demonstrated a greater percentage of multiparous cows in the OV-8x1 treatment had complete luteal regression compared with multiparous Ov-7x1 cows, whereas treatments were equally effective in primiparous cows. In contrast, results in experiment 2 revealed nearly 100% of cows in the Ov-7x2 treatment receiving the second dose of $PGF_{2\alpha}$ had complete luteolysis. In both experiments, when the status of luteal function before PGF treatment was examined, the treatments were equally effective in causing complete luteal regression. Pregnancy rates, however, did not differ among treatments indicating that any of the three treatments will likely produce similar pregnancy outcomes with the flexibility of applying either the 7- vs. 8-day treatments.

Introduction

Fixed-time insemination programs have been adopted by dairy managers to facilitate 100% artificial insemination submission rate and reduce the dependence on the detection of estrus. Adoption of various on-farm reproductive technologies increased rapidly in just 7 years based on surveys conducted in 2006 and repeated in 2013 in 17 of the major U.S. dairy states representing 80% of the dairy operations and more than 80% of the dairy cows (National Health Monitoring Survey, Animal Plant Health Inspection Service, USDA). More than 12% of dairy operations used a timed AI program at first services and more than 21% used such programs to resynchronize ovulation in cows diagnosed not pregnant. The original 7-day Ovsynch program (GnRH-1 [day 0] - 7 days - $PGF_{2\alpha}$ - 56 hours - GnRH-2 - 16 hours - AI) has been modified by further research.

Maximal pregnancy rate achieved with the Ovsynch program has at least 3 limiting factors: (1) failure of GnRH-1 to induce ovulation of the dominant follicle to initiate a new follicular wave; (2) complete luteolysis after a single dose of $PGF_{2\alpha}$; and (3) failure to induce ovulation after G-2. The proportion of cows ovulating after GnRH-1 was 64% (range of 23 to 96%) and varied by stage of the estrous cycle. Ovulation after GnRH-1 is maximized when the Ovsynch program is initiated between days 5 and 12 of the estrous cycle and this is accomplished by various presynchronization programs (Presynch, PG-3-G, Double Ovsynch, and G-6-G).

Cows exposed to presynchronization and that also ovulated after GnRH-1 generally have a greater pregnancy rate than cows that did not ovulate. Although it is assumed that the latter responses are associated with increased circulating progesterone from the newly formed corpus luteum (CL), during growth of the preovulatory follicle, the progesterone concentration at $PGF_{2\alpha}$ may not differ between cows with 1 older CL compared with cows with an older and new CL

(2 CL). Nevertheless, cows that ovulated after GnRH-1 and formed a new CL are more likely to have an incomplete luteolysis than cows that did not ovulate because a CL less than 7 days old is resistant to regression after a single standard dose of $PGF_{2\alpha}$.

Attempts have been made to prevent incomplete CL regression by either increasing the dose of $PGF_{2\alpha}$ or the frequency of $PGF_{2\alpha}$ treatments. Increasing the dose of $PGF_{2\alpha}$ (cloprostenol) from 500 to 750 μg for cows submitted to a 7-day Ovsynch protocol has increased the luteolytic risk, but this effect was only observed in multiparous cows. Inclusion of a second $PGF_{2\alpha}$ treatment 24 h after the first $PGF_{2\alpha}$ (days 7 and 8 after GnRH-1) increased the percentage of cows with complete luteolysis from 80 to 97%. Furthermore, a recent meta-analysis demonstrated a clear benefit of an additional $PGF_{2\alpha}$ treatment during the Ovsynch program on luteal regression (+11.6 percentage units) and on pregnancy rate (+4.6 percentage units).

We hypothesized that a single standard dose of $PGF_{2\alpha}$ injected on day 8 instead of day 7 after GnRH-1 increases the proportion of cows with complete luteolysis and increases the synchronization risk compared with control cows receiving a standard dose on day 7. We included a treatment with both standard doses of $PGF_{2\alpha}$ as a positive control. Therefore, our objective was to evaluate the effect of a single standard dose of $PGF_{2\alpha}$ treatment on day 7, day 8, or on both days on the luteolytic and synchronization risks in lactating Holstein cows inseminated at first service after a standard presynchronization program.

Experimental Procedures

Experiment 1

Lactating Holstein-Friesian cows (n = 347) in one commercial herd located in the southeast area of the Republic of North Macedonia were enrolled in the study. Cows were housed in free-stall barns, and fed a total mixed ration (TMR) once daily to meet or exceed requirements for lactating cows producing 30 L of milk per day. Cows were milked twice daily and had free access to water.

Starting at day 35 ± 3 postpartum, all cows were scored for body condition (BCS) on a five-point scale (1 = emaciated and 5 = obese). Estrous cycles were presynchronized using the PG-3-G scheme (Figure 1). The presynchronization scheme consisted of a 25-mg i.m. injection of $PGF_{2\alpha}$ (PrePG; 5 mL Lutalyse) followed by an i.m. injection of GnRH (8 μg Buserelin [GnRH agonist], PreGnRH; 2 mL of Receptal [GnRH agonist]) 3 days later. Ten days after the PrePG injection, an Ovsynch program was initiated (GnRH-1 – 7 days – $PGF_{2\alpha}$ – 56 hours – GnRH-2 – 16 hours – timed AI) and cows were allocated randomly to two treatments: (1) Ov-7x1 (n = 155) with an injection of $PGF_{2\alpha}$ on day 7 or (2) Ov-8x2 (n = 148) with an injection of $PGF_{2\alpha}$ on day 8 after GnRH-1, respectively. Cows in Ov-8x1 were inseminated 1 day later than cows in Ov-7x1. Pregnancy was diagnosed by transrectal ultrasonography.

Transrectal ultrasonography was conducted at GnRH-1 to map ovarian structures and at $PGF_{2\alpha}$ to assess ovulation in response to GnRH-1 (Figure 1). Blood samples to assess concentrations of progesterone were collected at PrePG, GnRH-1, $PGF_{2\alpha}$, at 72 hours after $PGF_{2\alpha}$ (at timed AI), and 7 days after timed AI from the coccygeal vein or artery for later measurement of progesterone.

Complete luteolysis was defined to occur when progesterone was ≥ 1 ng/mL before $PGF_{2\alpha}$ and ≤ 0.3 ng/mL 72 h later. In addition, when examining the effect of luteolysis, progesterone concentrations at $PGF_{2\alpha}$ were determined based on the luteal status: (1) cows with only a new CL (cows without a CL at GnRH-1 but ovulated after GnRH-1); (2) cows with an older CL (same CL that was detected at G-1); or (3) both a new and old CL.

Experiment 2

We enrolled lactating 454 Holstein cows weekly for 90 weeks at the Kansas State University Dairy Teaching and Research Center, Manhattan, KS. Cows were housed in free stalls with overhead roofs and fed a TMR twice or thrice (summer) daily calculated to meet nutrient requirements for lactating dairy cows producing 110 lb of 3.5% milk. The diet consisted of alfalfa hay, corn silage, triticale silage, soybean meal, whole cottonseed, ground corn grain, corn-gluten feed, vitamins, and minerals. Cows were milked thrice daily.

Beginning at postpartum day 52 ± 3, estrous cycles were presynchronized using PG-3-G protocol as in experiment 1 (Figure 1). The scheme consisted of a 25-mg i.m. injection of $PGF_{2\alpha}$ (Pre-PG; 2 mL Lutalyse HighCon) and 3 days later a 100-μg i.m. injection of GnRH (Pre-GnRH; 2 mL Factrel). Seven days after the Pre-GnRH treatment in weekly clusters of cows calving between August 2018 through April 2020 and stratified by parity (primiparous vs. multiparous), cows were assigned randomly to 3 treatments: (1) Ov-7x1 (n = 150): GnRH - 7 d - $PGF_{2\alpha}$ - 56 h - GnRH-2 - 16 h - timed AI; (2) Ov-8x1 (n = 149): GnRH-1 - 8 d - $PGF_{2\alpha}$ - 56 hours - GnRH-2 - 16 hours - timed AI; or (3) Ov-7x2 (n = 153): GnRH-1 - 7 d - $PGF_{2\alpha}$ - 24 h - $PGF_{2\alpha}$ - 32 hours - GnRH-2 - 16 hours - timed AI (Figure 1). Onset of the presynchronization treatments occurred 1 d earlier so cows could receive assigned treatments and AI on the same day in each cluster (73 ± 3 days in milk).

Transrectal ultrasonography was conducted at GnRH-1 and before the first or only $PGF_{2\alpha}$ treatment. Ovaries were scanned by transrectal ultrasonography to determine the number and location of CL and also the number and diameter of all follicles ≥ 10 mm (mapped and recorded) as in experiment 1.

Blood samples were collected before the first or only $PGF_{2\alpha}$ treatment and on the day of insemination (70 to 72 hours after the first or only $PGF_{2\alpha}$ treatment). Complete luteolysis was assessed as in experiment 1.

Results and Discussion

Experiment 1

Mean number of lactations was 1.9 ± 1.1 with 43.8% of cows in their first lactation. Median days in milk (DIM) at AI were 68 d ranging from 52 to 80 d with a mean ± standard deviation (SD) of 67.7 ± 6.3 d. Herd milk production averaged approximately 66 lb per cow per day.

Progesterone concentrations before GnRH-1, PGF_{2a}, and AI by treatment are in Figure 2A. Delaying luteolysis by 1 day did not increase progesterone concentrations before PGF_{2a}, and progesterone did not differ between treatments before GnRH-1 or PGF_{2a}. Body condition affected progesterone concentrations before PGF_{2a} as cows with BCS ≥ 2.75 had greater (P < 0.01) progesterone than cows with BCS < 2.75 (8.0 ± 0.3 vs. 6.4 ± 0.3 ng/mL), respectively. In contrast, at the time of AI, regardless of parity, concentrations of progesterone did not different for cows receiving the Ov-7x1 treatment (0.65 ± 0.2 ng/mL), whereas progesterone was less (P < 0.05) in multiparous than primiparous cows (0.45 ± 0.2 vs. 0.17 ± 0.2 ng/mL) receiving the Ov-8x1 treatment.

As expected, progesterone concentrations differed depending on CL status before PGF_{2a}. Cows bearing only a new CL that formed after ovulation in response to GnRH-1 had the smallest concentration and cows with both an older and new CL had the largest concentration resulting in mean progesterone concentrations that differed (P < 0.01) among the three luteal status groups (Figure 2B).

Our hypothesis was that lengthening the period between GnRH-1 and PGF_{2a} by 1 day would increase the proportion of cows with complete luteolysis by the time of AI because new CL forming after GnRH-1 would be 24 hours older and more likely to respond to a single dose of

PGF_{2a}. Of the 347 cows assigned to treatment, 87.9% were cyclic by the onset of treatment, leaving 303 cows to test this hypothesis. More ($P = 0.02$) multiparous than primiparous cows had complete luteolysis and more ($P < 0.05$) cows receiving the Ov-8x1 treatment had complete luteolysis than cows in the Ov-7x1 treatment (Table 1). An interaction ($P = 0.02$) of treatment and parity also was detected. Although complete luteolysis did not differ between treatments in primiparous cows, more ($P = 0.02$) multiparous cows had complete luteolysis when treated with Ov-8x1 than Ov-7x1 treatment (Table 1). Treatments were equally effective in causing complete luteolysis in cows with only a new CL at the time of PGF_{2a} (87.5%), an older CL (79.0%), or both (77.7%).

Pre-treatment cyclicity tended ($P = 0.10$) to affect pregnancy rate (23.8 vs. 36.8%) in anovular vs. cycling cows, respectively. Neither treatment nor parity, however, affected pregnancy rate (Table 2). Pregnancy rate in cows with a body condition score < 2.75 tended ($P = 0.09$) to be less in Ov-8x1 vs. Ov-7x1 cows (30.1 vs. 39.4%), respectively, whereas the reverse occurred in cows with body condition score ≥ 2.75 (40.9 vs. 31.7%), respectively.

Experiment 2

Mean number of lactations was 1.9 ± 1.1 with 44% of cows in their first lactation. Median DIM at AI was 68 d ranging from 52 to 80 d with a mean ± SD of 67.7 ± 6.3 d. Rolling herd average was 31,324 ± 458 lb during the experimental period.

Delaying luteolysis by 1 day did not increase progesterone concentrations before PGF_{2a} in the Ov-8x1 treatment. In fact, concentrations of progesterone were less ($P = 0.06$) in the cows treated with Ov-8x1 compared with the other two treatments (Figure 3A). Furthermore, progesterone concentrations were greater ($P < 0.001$) in primiparous cows compared with the multiparous cows (5.2 ± 0.2 vs. 4.3 ± 0.2 ng/mL).

As in experiment 1, progesterone concentrations differed depending on CL status before PGF_{2a}. Cows bearing only new CL had the smallest concentration and cows with both an older and new CL had the largest concentration resulting in mean progesterone concentrations that differed ($P < 0.01$) among the three luteal status groups (Figure 3B).

Cows receiving the second dose of PGF_{2a} had nearly perfect complete luteolysis, which was greater ($P < 0.05$) than that observed in cows receiving the other two treatments. Although not different, complete luteolysis was numerically greater in primiparous than multiparous cows. Complete luteolysis did not differ among cows having different CL status: new CL (91.7%), old CL (95.1%), and both new and old CL (93.3%).

Cows not cycling (n = 18) had numerically few pregnancies than cycling cows (n = 429; 27.8 vs. 41.7%), respectively. Neither treatment nor parity affected pregnancy rate (Table 4). Some variation in pregnancy rates occurred during each season with fall and winter (43.9%) tending ($P = 0.08$) to be greater than pregnancy rate during spring and summer (36.3%).

The present study aimed to determine the efficacy of injecting $PGF_{2\alpha}$ on day 8 instead of day 7 to produce more complete luteal regression in the standard Ovsynch protocol in lactating dairy cows. Overall, the results in experiment 1 in Holstein-Friesian cows demonstrated a greater percentage of multiparous cows in the OV-8x1 treatment had complete luteal regression compared with Ov-7x1 cows, whereas treatments were equally effective in primiparous cows. In contrast, results in experiment 2 in Holstein cows, revealed nearly 100% of cows in the Ov-7x2 treatment receiving the second dose of $PGF_{2\alpha}$ 24 hours after the first dose, had complete luteolysis. In both experiments, when the status of luteal function before PGF treatment was examined, the treatments were equally effective in causing complete luteal regression.

The resulting pregnancy rates achieved were the most important outcomes of these experiments. Pregnancy rates did not differ among treatments, indicating that any of the three treatments will likely produce similar outcomes with the flexibility of the 7- vs. 8-day treatments.

Table 1. Percentage of cows with complete luteolysis by 72 h after the PGF_{2a} treatment injection (time of AI) in primiparous and multiparous cows (experiment 1)[1]

	Parity		
Treatment[2]	Primiparous	Multiparous	Overall
Ov-7x1	75.4 (46/61)	75.5 (71/94)	75.5[a] (117/155)
Ov-8x1	74.0 (57/77)	93.0 (66/71)	83.1[b] (123/148)
Overall	74.6[a] (103/138)	83.0[b] (137/165)	79.2 (240/303)

[a,b] Means bearing different superscript letters differ ($P < 0.05$). A treatment by parity interaction ($P = 0.03$) also was detected.
[1] Complete luteolysis was defined to occur when progesterone was ≥ 1 ng/mL before $PGF_{2\alpha}$ treatment and ≤ 0.3 ng/mL 72 h later (time of AI). The table excludes 44 cows without a CL at $PGF_{2\alpha}$ treatment having progesterone < 1 ng/mL.
[2] See Figure 1.

Table 2. Pregnancy rates (%) in primiparous and multiparous cows at first AI after treatment (experiment 1)

	Parity		
Treatment[1]	Primiparous	Multiparous	Overall[2]
Ov-7x1	38.4 (26/68)	32.7 (35/107)	34.9 (61/175)
Ov-8x1	34.9 (29/83)	36.4 (32/88)	35.7 (61/171)
Overall[2]	36.4 (55/151)	34.4 (67/195)	35.3 (122/346)[3]

[1] See Figure 1.
[2] Neither parity nor treatment differences were detected.
[3] One cow was culled before pregnancy diagnosis.

Table 3. Percentage of cows with complete luteolysis by 72 h after the PGF_{2a} treatment injection (time of AI) in primiparous and multiparous cows (experiment 2)[1]

	Parity		
Treatment[2]	Primiparous	Multiparous	Overall
Ov-7x1	96.8 (60/62)	86.7 (65/75)	91.2[a] (125/137)
Ov-7x2	100 (60/60)	98.7 (74/75)	99.3[b] (134/135)
Ov-8x1	90.4 (47/52)	90.6 (58/64)	90.5[a] (105/116)
Overall	96.0 (167/174)	92.1 (197/214)	93.8 (364/388)

[a,b] Treatments differed ($P = 0.05$). No parity or treatment by parity interaction was detected.
[1] Complete luteolysis was defined to occur when progesterone was ≥ 1 ng/mL before $PGF_{2\alpha}$ treatment and ≤ 0.3 ng/mL 72 h later (time of AI). Excludes 66 cows without a CL at $PGF_{2\alpha}$ treatment having progesterone < 1 ng/mL.
[2] See Figure 1.

Table 4. Pregnancy rates in primiparous and multiparous cows (experiment 2)

	Parity		
Treatment[1]	Primiparous	Multiparous	Overall[2]
Ov-7x1	44.8 (30/67)	38.6 (32/83)	41.3 (62/150)
Ov-7x2	48.5 (32/66)	38.6 (32/83)	43.0 (64/149)
Ov-8x1	33.8 (22/65)	43.4 (36/83)	39.2 (58/148)
Overall[2]	42.4 (84/198)	40.2 (100/249)	41.1 (184/447)[3]

[1] See Figure 1.
[2] Neither parity nor treatment differences were detected.
[3] Seven cows were culled before pregnancy diagnosis.

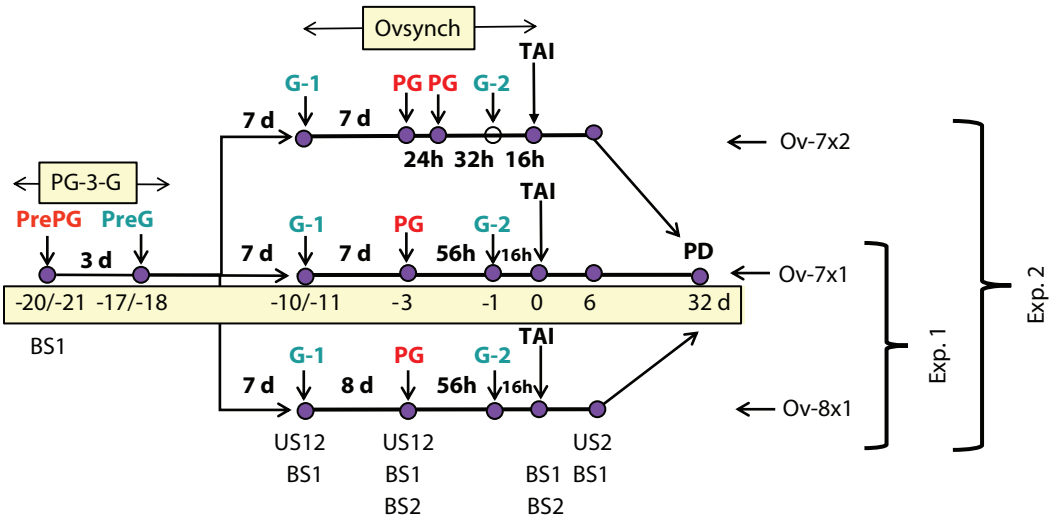

Figure 1. Experimental scheme for experiments 1 and 2. Ovulation was presynchronized in all cows before treatments were applied to facilitate first AI after calving. In both experiments presynchronization consisted of PG-3-G presynchronization (Pre-PGF_{2a} [PrePG] – 3 days – Pre-GnRH [PreG]). Seven days after PreG, cows were treated with either Ovsynch (Ov-7x1 or Ov-8x1; GnRH-1 [G-1] – 7 or 8 days – $PGF_{2\alpha}$ – 56 hours – GnRH-2 [G-2] – 16 hours – timed AI [TAI]) in experiment 1. In experiment 2, an additional Ovsynch treatment was included (Ov-7x2) in which two doses of PGF_{2a} were administered 24 hours apart followed by G-2 in 32 hours and timed AI 16 hours after G-2. Pregnancy diagnosis (PD) occurred 30 to 32 days after timed AI. US12 = transrectal ultrasonography of ovaries (1 or 2 designates experiment 1 or 2, respectively); BS = blood sample.

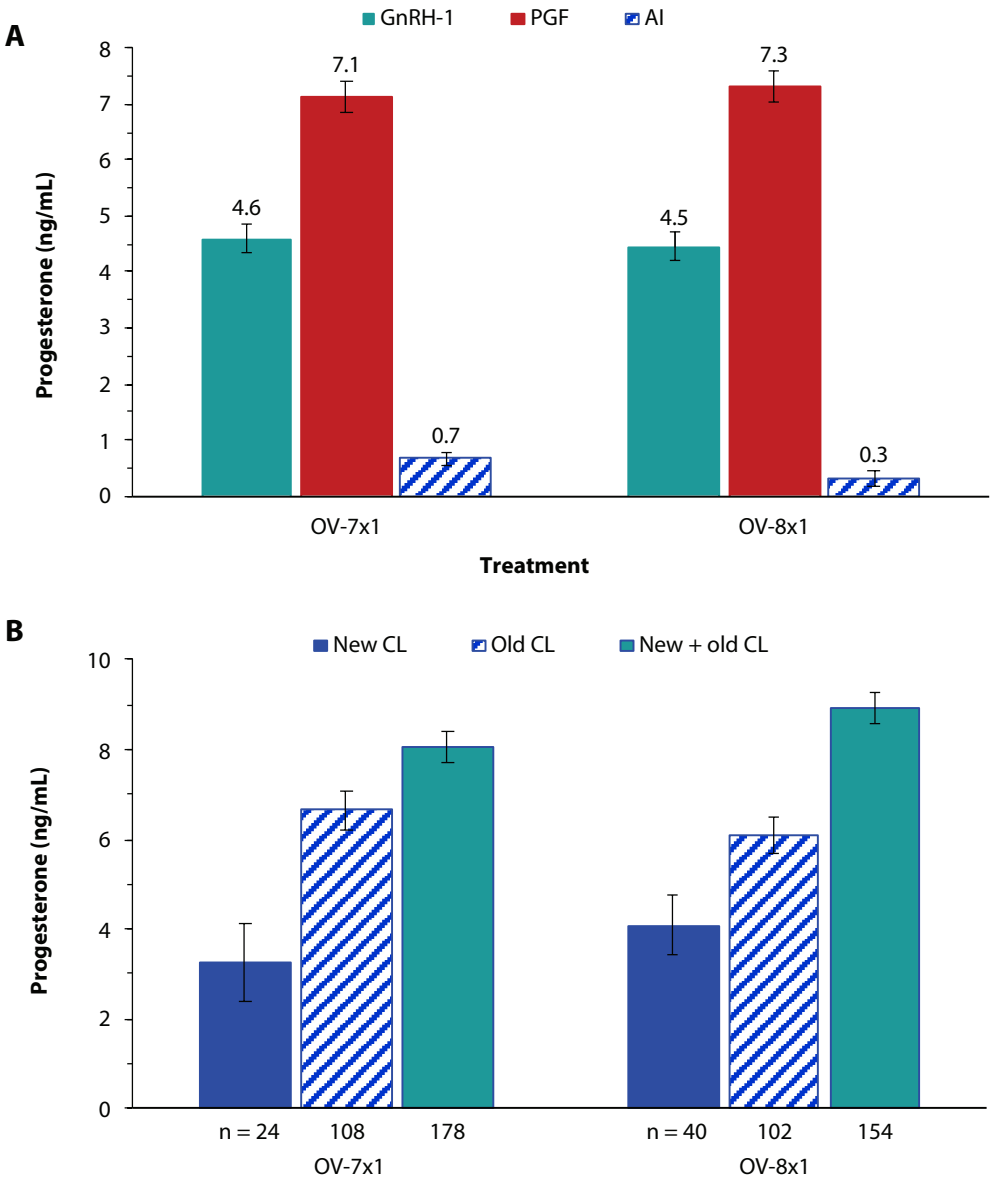

Figure 2. (A) Concentrations of progesterone (LSM ± SEM) before GnRH-1, $PGF_{2\alpha}$ (PGF), and timed AI in experiment 1. (B) Concentrations of progesterone (LSM ± SEM) before $PGF_{2\alpha}$ treatment injection in cows receiving the Ov-7x1 or Ov-8x1 treatment. Cows were classified at the time of $PGF_{2\alpha}$ treatment injection as having a new CL (formed in response to ovulation after GnRH-1), an older CL existing at GnRH-1 and present at $PGF_{2\alpha}$ or both a new and older CL.

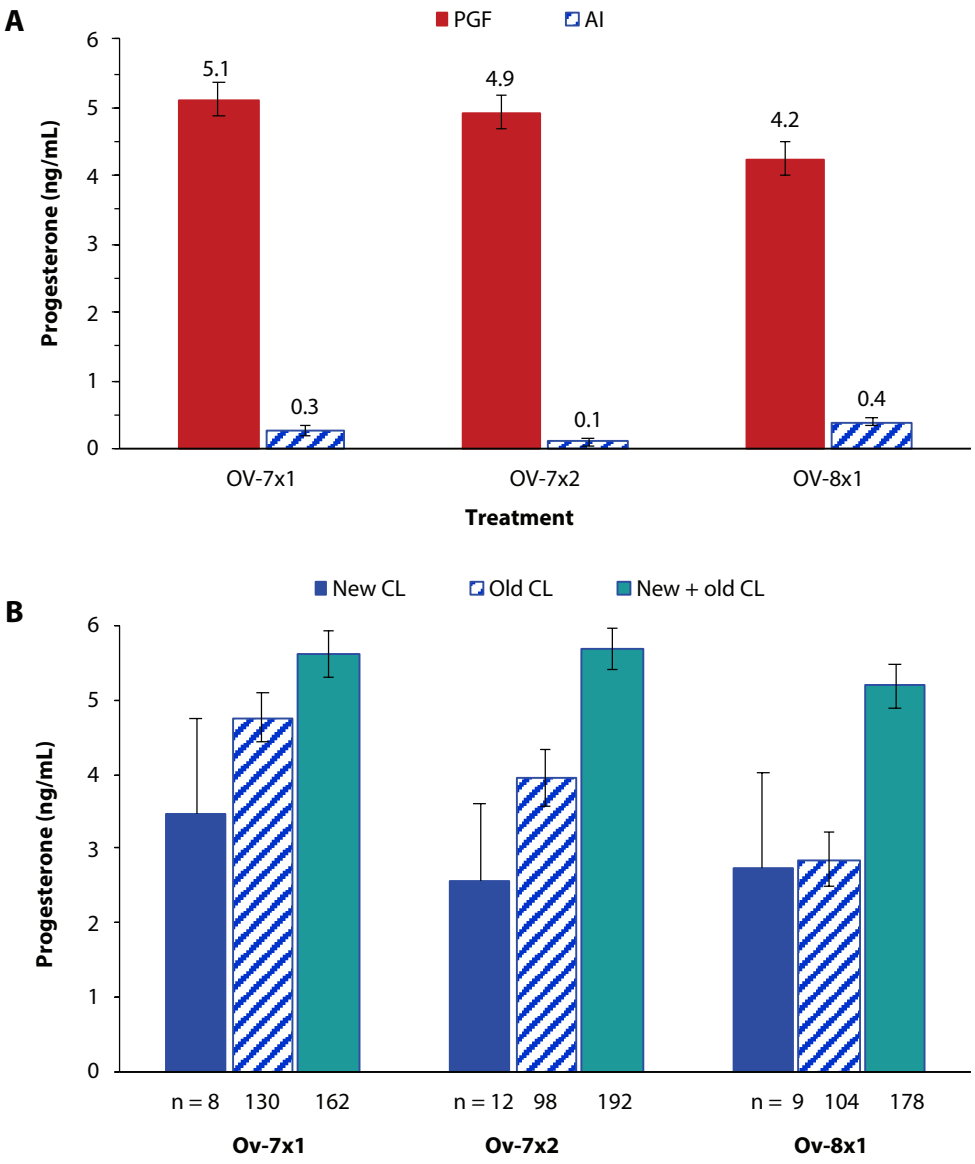

Figure 3. (A) Concentrations of progesterone (LSM ± SEM) before PGF2α (PGF) and timed AI in experiment 2. (B) Concentrations of progesterone (LSM ± SEM) before $PGF_{2\alpha}$ treatment injection in cows receiving the Ov-7x1, Ov-7x2, or Ov-8x1 treatment. Cows were classified at the time of $PGF_{2\alpha}$ treatment injection as having a new CL (formed in response to ovulation after GnRH-1), an older CL existing at GnRH-1 and present at $PGF_{2\alpha}$ or both a new and older CL.

Resynchronizing Returns to Estrus after a Prior Insemination

J.S. Stevenson and J.A. Sauls-Hiesterman

Summary

In two experiments, GnRH and a progesterone insert (CIDR) were applied to cows after insemination (day 0) to reprogram or synchronize the returning estrus of cows that failed to conceive to that previous insemination. The combination of GnRH (day 14) and a CIDR insert (days 17 to 24) in experiment 1 (n = 347 cows) increased the proportion of nonpregnant cows returning to estrus before pregnancy diagnosis on day 32 by 7.4 percentage points, increased the synchrony of their return by 24.4 percentage points, but delayed that return by 2.3 days compared with controls. In experiment 2 (n = 863 cows), use of GnRH alone (day 7), a CIDR insert alone (days 14 to 21), or in combination, failed to increase the proportion of nonpregnant cows in estrus before pregnancy diagnosis on day 32, but cows receiving the CIDR insert had increased synchrony of estrus by 24 to 34 percentage points compared with cows that did not receive a CIDR insert. In both experiments, the treatment of cows with GnRH or GnRH + CIDR insert increased the pretreatment pregnancy rate by 7.1 to 9.5 percentage points. We conclude that administering GnRH with or without a CIDR insert to resynchronize returns to estrus did not significantly improve the proportion of nonpregnant cows reinseminated before pregnancy diagnosis.

Introduction

Although applying various fixed-time insemination programs are critical components of reproductive management of non-pregnant cows, fewer cows would require such intervention if more cows were identified in estrus before pregnancy diagnosis. Detection of returns to estrus from 18 to 24 days after a prior insemination is a labor-intensive method to determine previous pregnancy failure in dairy cattle. Detection of spontaneous returns to estrus, however, is a substantial challenge for dairy herds, with approximately 40 to 60% of non-pregnant cows not reinseminated before pregnancy diagnosis. Efforts to increase the percentage of accurately diagnosed returns to estrus could reduce inter-insemination intervals and promote more efficient use of labor.

Attempts to resynchronize the first eligible estrus in previously inseminated cows have met with limited success because variation in the duration of the estrous cycle after a previous insemination is quite large. Dairy cows with two follicular waves per cycle have shorter estrous cycles, with the ovulatory follicle being both larger and older compared with cows having three follicular waves before insemination. Furthermore, in cows with three follicular waves, luteal function was extended, the peak in plasma progesterone occurred later in the estrous cycle, and conception rate was greater compared with two-wave cows. Estrus-cycle duration could be reprogrammed via GnRH administration to ovulate the second-wave dominant follicle and cause cows to develop a third follicular wave before returning to estrus (experiment 1). Administering GnRH earlier on day 7, for example, could ovulate a first-wave dominant follicle and form an accessory corpus luteum (experiment 2).

Applying a progesterone insert on day 13 or 14 for 7 days, neither harmed nor benefitted ongoing pregnancies, but synchronized returns to estrus in nonpregnant cows. Reprogramming follicular waves to three per cycle in the presence of exogenous progesterone administered at the appropriate time (experiment 1) or inducing an accessory corpus luteum (experiment 2) could synchronize the return to estrus for cows previously inseminated and potentially improve conception rate for cows that are reinseminated.

The hypothesis tested in two experiments was that the application of designed treatments to synchronize estrus in nonpregnant previously inseminated cows leads to reduced inter-insemination intervals and enhanced estrus-detection of nonpregnant cows before pregnancy diagnosis on day 32 after insemination.

Our objective was to reprogram follicular waves in previously inseminated cows by exposing them to GnRH and then controlling return to estrus with exogenous progesterone. We determined the inter-insemination intervals of cows returning to estrus before and after pregnancy diagnosis on day 32 that followed a prior insemination and a subsequent resynchronization treatment. As part of those treatments pre- and post-treatment pregnancy rates also were monitored.

Experimental Procedures

We enrolled lactating Holstein cows in two experiments at the Kansas State University Dairy Teaching and Research Center, Manhattan, KS. Cows were housed in free stalls with overhead roofs and fed a total mixed diet twice or thrice (summer) daily, calculated to meet nutrient requirements for lactating dairy cows producing 110 lb of 3.5% milk fat. The diet consisted of alfalfa hay, corn silage, triticale-sweet clover silage, soybean meal, whole cottonseed, ground corn grain, corn-gluten feed, vitamins, and minerals. Cows were milked thrice daily.

Experiment 1

To reprogram follicular waves from two to three in conjunction with the application of a progesterone insert after a previous AI (day 0), 347 lactating cows were assigned randomly to two treatments during 13 months (June 2017 through June 2018) in 48 weekly clusters. Treatments were: (1) control; or (2) GnRH + CIDR: 200-μg GnRH on day 14 plus a progesterone (1.38 g) insert applied from d 17 through 24. Criterion for enrollment on day 14 required each cow to have a corpus luteum greater than 20 mm in diameter with bright echogenicity consistent with a functional corpus luteum.

Cows were fit previously with CowManager SensOor ear tags (Agis, Harmelen, the Netherlands) to monitor estrus. Determinations of estrus were diagnosed by the software "heat alerts." Insemination was based on the activity monitor alerts in addition to visual observation of standing-to-be mounted activity. Pregnancy diagnosis occurred weekly (day 32; range in days since insemination was days 30 to 36). Cows diagnosed not pregnant at day 32 (n = 230) bearing a CL > 20 mm were treated immediately i.m. with a Short Synch procedure consisting of prostaglandin F_{2a} (2 mL Lutalyse HighCon) and a second dose administered 24 hours later, followed by GnRH (2 mL Factrel) at 56 h after the not-pregnant diagnosis, and insemination 12 to 16 hours later.

Experiment 2

A second experiment was conducted during 24 months (July 2018 through June 2020) to examine at each component of a GnRH + CIDR combination treatment. Lactating dairy cows (n = 863) were assigned randomly to four treatments on day 7 after insemination. Treatments were: (1) control; (2) GnRH: 100 μg GnRH (2 mL Factrel) on day 7; (3) CIDR: insertion of a CIDR on day 14 for 7 days and removed on day 21; or (4) GnRH + CIDR (G + C): combination of the latter two treatments. As for experiment 1, eligibility for enrollment on day 7 required each cow to have a CL greater than 20 mm in diameter with bright echogenicity consistent with a functional CL.

Cows were fit previously with CowManager SensOor ear tags to monitor estrus. Inseminations and pregnancy diagnosis occurred as described for experiment 1. Cows diagnosed not pregnant at day 32 (n = 544) bearing a CL > 20 mm were treated immediately i.m. with a Short Synch procedure as in experiment 1.

Results and Discussion

Experiment 1

Simple univariate characteristics of cows enrolled in experiment 1 are as follows. The average number of inseminations at enrollment was 2.0 ± 1.7 (mean ± SD; range of 1 to 7) with an average 106 ± 49 days in milk (DIM; range of 63 to 280). Body condition scores (1 = thin and 5 = obese) averaged 2.6 ± 0.4 (range of 1.50 to 4.00). Parity of enrolled cows ranged from 1 to 5 with an average of 2.0 ± 1.1 (mean ± SD) and the pre-enrollment DHI-test day energy-corrected milk averaged 49.0 ± 9.7 kg/d (108 ± 21.4 lb) with a range of 25.4 to 70.3 kg/day (61.1 to 184.8 lb).

Although numerically greater by 7.3 percentage points, the pretreatment pregnancy rate of cows receiving either the GnRH + CIDR treatment did not differ ($P = 0.15$; Table 1) from controls, but was greater ($P = 0.02$) during the colder than hotter months (39.0 vs. 26.8%). Embryo loss did not differ between controls (3/51; 5.9%) and cows exposed to the GnRH + CIDR treatment (7/66; 10.6%).

The proportion of cows detected in estrus before pregnancy diagnosis was only numerically greater ($P = 0.13$) for GnRH + CIDR compared with control cows (59.5 vs. 52.1%), respectively. Greater synchrony of estrus occurred as more ($P < 0.01$) GnRH + CIDR cows (72.7%) were in estrus on days 26 to 27 compared with only 48.3% of controls on days 21 to 24 (Figure 1A). The pattern of distribution of cows detected in estrus differed ($P < 0.05$) between treatments with different peak and median days to estrus as shown in the survival curve (Figure 1B). Proportion of returning cows was greater ($P = 0.02$) for primiparous than for multiparous cows (65.4 vs. 48.7%), respectively, but an interaction of treatment and parity also was detected. Although treatment did not affect the proportion of multiparous cows returning to estrus compared with controls (46.2 vs. 51.2%), respectively; among primiparous cows, GnRH + CIDR increased the percentage of returns to estrus compared with the control (76.2 vs. 52.8%). The risk of a longer inter-insemination interval was greater ($P < 0.001$) for GnRH + CIDR-treated cows than for controls (hazard ratio = 1.985; 1.39 to 2.84).

In a subset of 100 cows detected in estrus before pregnancy diagnosis, and based on the CowSensOor accelerometer data, duration of estrus was greater ($P < 0.05$) in GnRH + CIDR cows than in control cows (14.3 ± 0.7 vs. 12.5 ± 0.7 h). In contrast, intensity of estrus was only numerically ($P = 0.13$) greater for GnRH + CIDR than control cows (5.5 ± 0.2 vs. 5.1 ± 0.2 units). For cows detected in estrus before pregnancy diagnosis, the inter-insemination interval was greater ($P < 0.01$) in GnRH + CIDR than control cows, but the subsequent pregnancy rate did not differ between treatments (Table 1).

For cows not detected in estrus before pregnancy diagnosis and diagnosed not pregnant, neither the inter-insemination interval nor the subsequent pregnancy rate after a fixed-time insemination differed between treatments (Table 1). Overall, for all cows regardless of when reinsemination occurred relative to pregnancy diagnosis, neither inter-insemination interval nor pregnancy rate differed between treatments (Table 1). The overall inter-insemination interval was greater ($P = 0.03$) for multiparous than primiparous cows (31.5 ± 0.7 vs. 29.4 ± 0.7 d), respectively. No interactions of parity and treatment were detected for any of the outcomes in Table 1.

Experiment 2

Average number of inseminations at enrollment was 1.7 ± 3.7 (mean ± SD; range of 1 to 8) and cows averaged 108 ± 50 DIM (range of 57 to 367). Body condition scores averaged 2.4 ± 0.4 (range of 2.0 to 4.0). Parity of enrolled cows ranged from 1 to 6 with an average of 2.0 ± 1.1 (mean ± SD) and the most recent DHI-test day energy-corrected milk averaged 103.5 ± 17.9 lb/day with a range of 56 to 155 lb/day.

Cows treated with GnRH on d 7 (GnRH and GnRH + CIDR treatments) tended ($P = 0.08$) to have greater pretreatment pregnancy rate (41.3%; 182/441) compared with cows not treated with GnRH (35.2%; 155/440). Neither the main effect of CIDR nor the interaction between GnRH and CIDR affected pretreatment pregnancy rate (Table 2). In addition, season ($P = 0.32$) and parity ($P = 0.57$) did not affect pregnancy rate. Embryo loss resulting from the pretreatment inseminations did not differ between controls (8/74; 10.8%), CIDR (2/81; 2.5%), GnRH (7/89; 7.9%), and cows exposed to the GnRH + CIDR treatment (7/93; 7.5%).

The proportion of cows detected in estrus before pregnancy diagnosis on day 32 did not differ among treatments even though the percentages ranged from 50 to 60% (Table 2). Greater synchrony of estrus was detected in those cows receiving a CIDR insert as more cows ($P < 0.05$) were in estrus on d 21 through 24 compared with cows not receiving a CIDR insert (Figure 2A). Pattern of distribution of nonpregnant cows returning to estrus after the pretreatment insemination did not differ between cows receiving a CIDR insert (CIDR and GnRH + CIDR treatments) and did not differ between controls and GnRH-treated cows, but distributions of non-CIDR-treated cows differed from CIDR-treated and was delayed as shown in the survival curve (Figure 2B). Proportion of cows detected in estrus before pregnancy diagnosis was greater ($P = 0.04$) for primiparous than for multiparous cows (61.2 vs. 52.7%), respectively. Furthermore, more cows tended ($P = 0.06$) to be detected in estrus during the colder months (58.1%; 194/334) compared with the hotter months (53.2%; 107/201).

The risk of a longer inter-insemination interval was not different ($P = 0.34$) for cows exposed to the CIDR compared with those not treated with a CIDR (HR = 0.89; 0.71 to 1.12). Median inter-insemination interval was 23 days (mean = 23.3 ± 0.2 days) for cows receiving a CIDR insert and 22 days (mean = 22.5 ± 0.3 days) for cows not receiving the CIDR insert.

For cows detected in estrus before pregnancy diagnosis, the inter-insemination interval was approximately 1 d shorter ($P = 0.04$) for cows not receiving a CIDR insert compared with those treated with a CIDR insert, but subsequent pregnancy rate did not differ between treatments (Table 2). In addition, the inter-insemination interval tended ($P = 0.07$) to be shorter in primiparous compared with multiparous cows (22.6 ± 0.5 vs. 23.4 ± 0.3 days), respectively. Moreover, pregnancy rate of cows detected in estrus was greater ($P = 0.04$) in primiparous cows (29.5%; 25/84) than in multiparous cows (18.9%; 27/144).

For cows not detected in estrus before pregnancy diagnosis and diagnosed not pregnant, the inter-insemination interval did not differ among treatments (Table 2), but the subsequent pregnancy rate after a fixed-time insemination was greater ($P = 0.04$) for cows in treatments that received GnRH (27.2%; 31/114) compared with cows not receiving GnRH (17.5%; 20/114). In addition, the inter-insemination interval for non-pregnant cows reinseminated after pregnancy diagnosis was shorter ($P = 0.02$) in primiparous compared with multiparous cows (36.2 ± 0.5 vs. 37.5 ± 0.4 days), respectively.

Overall, for all cows regardless of when reinsemination occurred relative to pregnancy diagnosis, neither inter-insemination interval nor pregnancy rate differed among treatments (Table 2). The pregnancy rate, however, tended ($P = 0.09$) to be greater in cows receiving GnRH (26.8%; 67/250) compared with cows not receiving GnRH (20.8%; 56/269). Overall, inter-insemination interval was shorter ($P < 0.01$) in primiparous compared with multiparous cows (27.9 ± 0.5 vs. 30.3 ± 0.5 days), respectively. In addition, the overall pregnancy rate for all reinseminated cows was greater ($P < 0.01$) in primiparous (28.4%; 61/215) compared with multiparous cows (20.4%; 62/304), respectively. No interactions of parity and treatment were detected for any of the outcomes in Table 2.

Discussion

In both experiments, previously inseminated cows treated with either GnRH alone or both GnRH + CIDR compared with the control had numerically improved pregnancy rates compared to the control by 7.3 percentage points (GnRH + CIDR in experiment 1), 7.1 percentage points (GnRH in experiment 2), and 9.5 (G + C in experiment 2) percentage points. This finding is consistent with other studies and shows a benefit of providing a second corpus luteum and more endogenous or supplemental progesterone during early pregnancy. The cost associated with applying both GnRH and a CIDR insert could vary from $12 to $14 per cow in addition to the labor cost. At a cost of $12 per cow and an increase of 8 pregnancies per 100 cows treated, the value of each pregnancy must be worth more than $66.67 per cow to cover production costs alone. Average value of a new pregnancy was $278 for a Holstein cow in the United States in 2006. The value of a new pregnancy increased with days in milk early in lactation but typically decreased later in lactation. Relatively high-producing cows and first-lactation cows reached greater values, and their values peaked later in lactation. The average cost of a pregnancy loss (abortion) was $555. The value of pregnancy and optimal breeding decisions for individual cows greatly depend on the predicted daily milk yield for the remaining period of lactation.

We numerically increased the percentage of nonpregnant cows detected in estrus (7.4 percentage points) before pregnancy diagnosis by the GnRH + CIDR treatment in experiment 1, but were unsuccessful in experiment 2 with any of the treatments administered at a different time. In contrast, in both experiments, returns to estrus were more synchronous with more cows in estrus at the same time. The inter-insemination intervals of cows in experiments 1 and 2 were increased by 2.3 days or 1 day, respectively, because of the progesterone supplementation.

In experiment 2, treatment with GnRH on day 7 resulted in improved pregnancy rates in cows that were detected not pregnant at day 32 and received the timed insemination 3 days after the not-pregnant diagnosis.

Conclusions

We did not prove our hypothesis that the designed treatments would increase the proportion of nonpregnant cows in estrus before the first pregnancy diagnosis after a prior insemination. Although nearly 60% of nonpregnant cows treated with GnRH + CIDR in experiment 1 were detected in estrus, the synchrony of their expressed estrus also was improved. Treatments in experiment 2 failed to increase the returns to estrus, but did increase the synchrony of those returns. Given the costs associated with such treatments, attempts to resynchronize returns to estrus in previously inseminated cows are not advisable.

Table 1. Pretreatment pregnancy rate, subsequent inter-insemination intervals, characteristics of estrus (LSM ± SEM), and pregnancy rate for cows returning to estrus before or after pregnancy diagnosis (experiment 1)

	Treatment[1]		
Item	Control	GnRH + CIDR	*P*-value
Pretreatment pregnancy rate, %	30.0 (51/170)	37.3 (66/177)	0.15
Detected in estrus,[2] %	52.1 (62/119)	59.5 (66/111)	0.13
Inter-insemination interval, d	24.0 ± 0.3	26.3 ± 0.3	<0.01
Return pregnancy rate, %	29.0 (18/62)	27.3 (18/66)	0.85
Not detected in estrus[3]			
Inter-insemination interval, d	37.0 ± 0.7 (57)	37.7 ± 0.9 (45)	0.57
Return pregnancy rate, %	22.8 (13/57)	26.7 (12/45)	0.64
Overall			
Inter-insemination interval, d	30.2 ± 0.7	30.7 ± 0.7	0.62
Pregnancy rate, %	26.1 (31/119)	27.0 (30/111)	0.80

[1]Cows were controls or treated with 200 μg GnRH on day 14 and received a CIDR insert between days 17 and 24 after insemination (d 0).
[2]Detected in estrus visually or by SensOor tags and reinseminated before pregnancy diagnosis on day 32.
[3]Not detected in estrus before pregnancy diagnosis on day 32 and reinseminated by fixed-time AI after a non-pregnancy diagnosis.

Table 2. Pretreatment pregnancy rate and inter-insemination interval (LSM ± SEM) and pregnancy risk after resynchronization with GnRH, progesterone, or both (experiment 2)

	Treatment[1]				*P*-value		
Item	Control	CIDR	GnRH	G + C	GnRH	CIDR	G × C
Pretreatment pregnancy rate, %	33.0 (74/224)	37.5 (81/216)	40.1 (89/222)	42.5 (93/219)	0.08	0.44	0.81
Detected in estrus,[2] %	59.5 (89/148)	55.6 (75/135)	50.0 (64/128)	59.7 (74/124)	0.59	0.59	0.15
Inter-insemination interval, days	22.3 ± 0.4 (88)	23.3 ± 0.4 (75)	22.8 ± 0.5 (64)	23.5 ± 0.4 (74)	0.46	0.04	0.70
Return pregnancy rate, %	25.3 (21/83)	20.8 (15/72)	29.0 (18/62)	24.3 (18/74)	0.52	0.36	0.89
Not detected in estrus[3]							
Inter-insemination interval, days	36.9 ± 0.5 (61)	37.4 ± 0.6 (60)	36.9 ± 0.6 (64)	36.2 ± 0.6 (50)	0.28	0.86	0.30
Return pregnancy rate, %	15.8 (9/57)	19.0 (11/58)	28.1 (18/64)	26.0 (13/50)	0.04	0.99	0.77
Overall							
Inter-insemination interval, days	28.2 ± 0.7 (149)	29.7 ± 0.7 (135)	29.8 ± 0.7 (128)	28.6 ± 0.7 (124)	0.76	0.82	0.06
Pregnancy rate, %	21.4 (30/140)	20.0 (26/130)	28.6 (36/126)	25.0 (31/124)	0.09	0.39	0.95

[1]Control cows were untreated, or cows were treated with 100 μg GnRH on day 7 post-AI, received progesterone supplementation (CIDR) from days 7 to 14 after insemination (day 0), or both GnRH and CIDR (G + C) treatments. Cows culled before reinsemination (2 controls, 0 CIDR, 5 GnRH, and 2 G + C cows) or before pregnancy diagnosis after reinsemination: (8 controls, 5 CIDR, 2 GnRH, and 0 G + C cows), respectively.
[2]Detected in estrus visually or by SensOor tags and reinseminated before pregnancy diagnosis on day 32.
[3]Not detected in estrus before pregnancy diagnosis on day 32 and reinseminated by fixed-time AI after a non-pregnancy diagnosis.

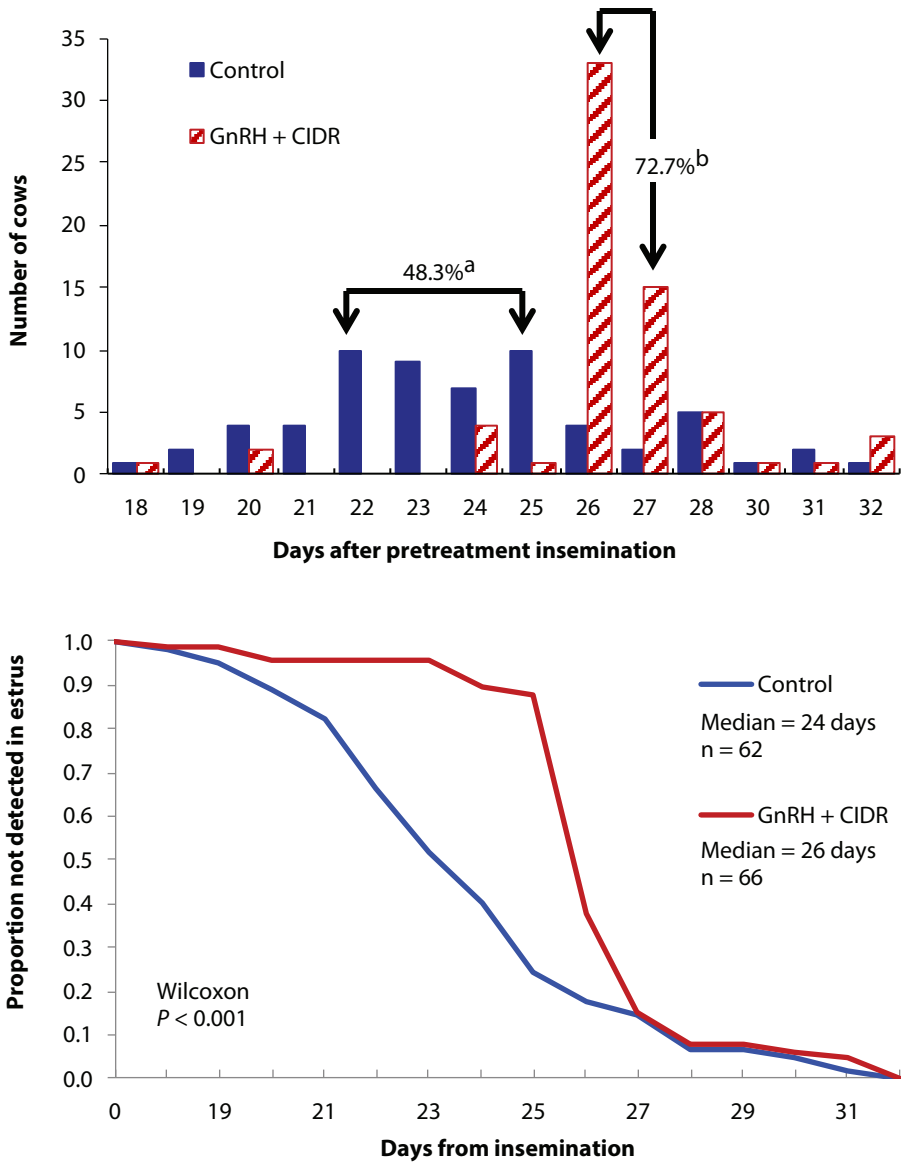

Figure 1. (A) Distribution of nonpregnant cows detected in estrus after a prior insemination and before pregnancy diagnosis on day 32 after insemination (d 0). Control cows were untreated or cows were treated with 200 μg GnRH on day 14 post-AI and received progesterone supplementation (CIDR insert) from days 17 through 24 after insemination. [a,b]Percentage in estrus on days 21 through 24 in the control differed ($P < 0.01$) from that in GnRH + CIDR cows in estrus on days 26 and 27. (B) Survival curve illustrating the proportion of cows not detected in estrus and reinseminated before pregnancy diagnosis on day 32 (experiment 1).

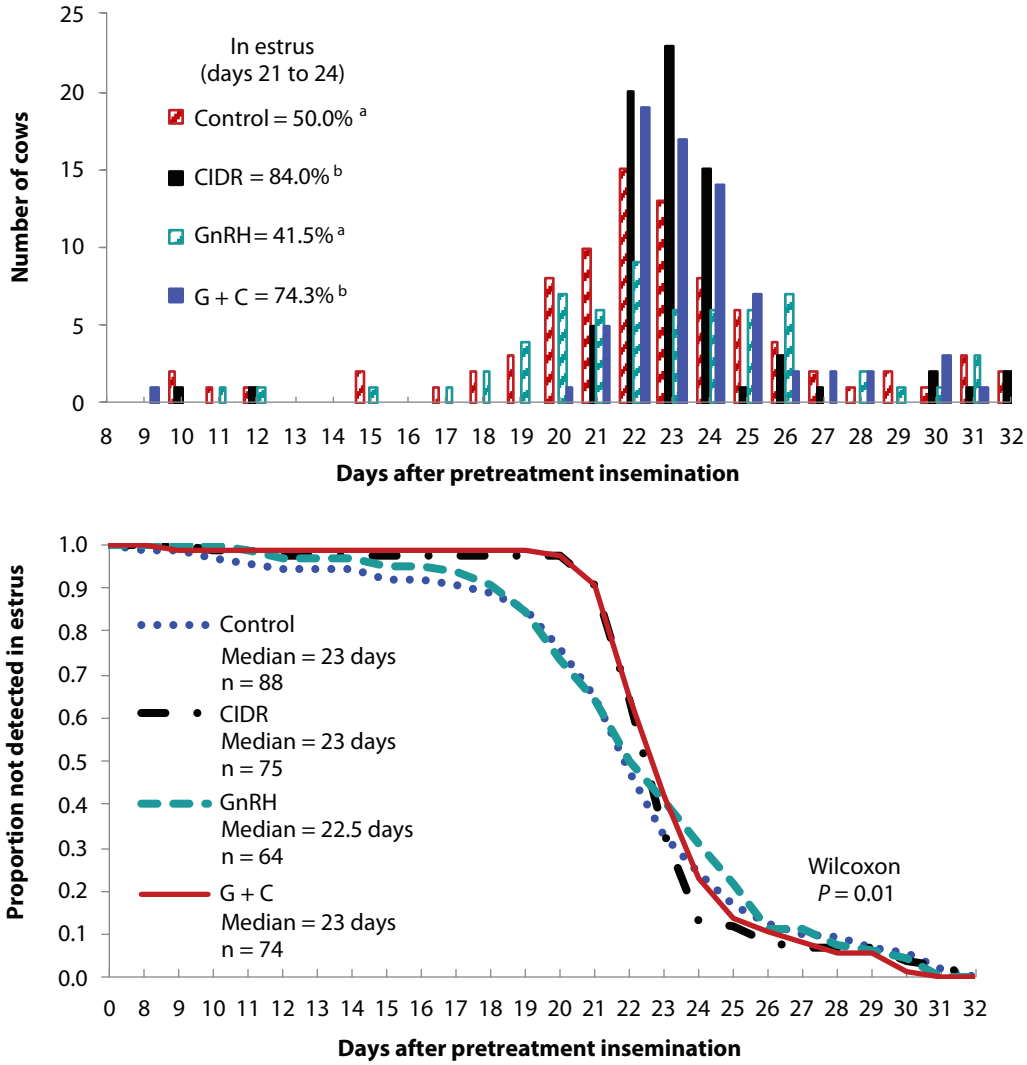

Figure 2. (A) Distribution of nonpregnant cows detected in estrus after the pretreatment insemination (d 0) and before pregnancy diagnosis on day 32 for controls, cows treated with 100 μg GnRH on day 7, cows receiving progesterone supplementation (CIDR) from days 7 through 14, or both GnRH and CIDR (G + C) treatments. [a,b]Percentage in estrus on days 21 through 24 differed ($P < 0.01$) among treatments. (B) Survival curve illustrating the proportion of cows detected in estrus and reinseminated before pregnancy diagnosis on day 32 (experiment 2).

Effects of Pre-Cutting Round Alfalfa Hay Bales on Forage Quality and Processing Time

W.E. Brown, E. Harms,[1] J. Heimsoth,[1] J. McGinnis,[1] C.I. Vahl,[2] B.J. Bradford, and M.J. Brouk

Summary

Round hay balers with knives that cut the hay as it enters the baling chamber reduce the particle size upon baling, and eliminate the need for a tub grinder. The objective of this study was to evaluate the effects of a round hay baler with knives on forage quality of alfalfa hay at baling and after storage, and the effects of the processing method on nutrient composition and particle size distribution. Alfalfa hay was baled (560 M Megawide HC[2], John Deere, Moline, IL) with knives every 4 inches (CUT; theoretical length of cut) or without knives (NORM). At baling and after 6 months of uncovered storage, bales were weighed, measured, and 10 core samples were obtained for nutrient analysis. Cores were separated into outer 6 inches and inner 6- to 18-inch segments to determine the depth of spoilage. After storage, particle size was reduced to approximately 4 inches using a mixer wagon for CUT (CUT-MIX) or a tub grinder for NORM (NORM-GRIND). Compared with NORM, CUT increased bale weight and density. Core depth interacted with storage timepoint whereby acid detergent fiber (ADF) concentration increased more for outer than inner cores from baling to the end of storage, with similar effects for lignin and 240-hour undigestible NDF. Compared with NORM, CUT increased concentrations of aNDF organic matter, ADF, and lignin, and decreased relative forage quality (RFQ). The CUT-MIX treatment increased time to reduce particle size, but decreased processing shrink by 6.1% compared with NORM-GRIND. Additionally, when compared with NORM-GRIND, CUT-MIX increased fiber content and decreased fiber digestibility, which may have been due to sampling error from longer particle size. In summary, CUT produced larger, more dense bales and increased fiber content slightly, and CUT-MIX decreased processing shrink but increased fiber content with additional longer particles after processing, which could be advantageous for physically effective fiber in ruminant diets. Further work should continue to evaluate leaf loss during baling, and options for processing and incorporating pre-cut hay into diets.

Introduction

The use of round hay bales in livestock feeding in North America is common, either through animals eating directly from the bale or caretakers incorporating hay into mixed diets delivered to the animals. If hay from round bales is to be incorporated into diets and delivered to animals, it must be disassembled to facilitate ease of mixing and to reduce particle size for stimulation of feed intake in high forage diets limited by gut fill. A common method for round bale disassembly is the use of tub grinders, which rapidly break down the bale and reduce hay particle size. While this method is relatively quick and effective, there are several drawbacks. First, a large amount of shrink occurs as high-value components of the feed drift away as small fragments into the environment, especially on windy days. Secondly, the ground hay typically is stored outdoors and exposed to moisture and wind for extended periods of time until it can be fed, with estimated losses incurred up to 20%. Finally, the use of a tub grinder requires specialized equipment, either purchased or contracted, creating an additional expense per ton of feed offered to the animal. The energy requirements of grinding also increase energy costs to reduce particle size.

[1] John Deere Corporation, Olathe, KS.
[2] Department of Statistics, College of Arts and Sciences, Kansas State University.

Round hay balers with vertical knives reduce the particle size of hay before entering the baling chamber, which potentially eliminates the need for tub grinders to break apart round bales before incorporation into animal diets. Hay produced in this manner is widely referred to as precut or presliced hay and provides unique feed management possibilities to prevent forage losses during preparation for mixed ration delivery. While precut hay baler models have been available from various equipment manufacturers for over two decades, little peer-reviewed data are available on forage produced in this manner. The data that are available focus on animal feeding behavior and production performance, forage quality, and fermentation of wrapped precut hay bales, and bale characteristics and power requirements for bale production.

In hay production, an estimated dry matter (DM) loss of 25% may occur between harvest and feeding, so maximizing nutrient retention from harvest to feed delivery is critical to ensure the nutritional requirements of the animal are met cost-effectively. Nutrient loss occurs from harvest through feeding of round hay bales, and precutting bales may eliminate the need for tub grinding. The objectives of this study were to 1) assess the effect of precutting alfalfa hay on forage quality at baling and after storage, and 2) determine the effects of post-storage processing method determined by bale production system on forage quality. We hypothesized that precutting alfalfa hay would have minimal impacts on forage quality at baling and through storage, and that precut bales would retain more nutrients during processing with a mixer wagon due to less leaf loss to the environment compared with grinding bales.

Experimental Procedures

This trial was conducted on four farms in south central Nebraska in the summer and winter of 2019 as a split-split-plot design. A John Deere 560 M Megawide HC[2] (John Deere Corporation, Moline, IL) was used to produce round alfalfa bales of either first or second cutting, with two farms for each cutting. Bales were produced using vertical knives spaced 4 inches apart between the pickup and the baling chamber (CUT) or with the knives retracted to produce a normal bale (NORM) and each treatment was applied to alternate bales as the baler moved through the field. Knives were not present on the outer 6 inches of the baler to preserve bale stability. There were 6 bales of each treatment produced on each farm for a total of 48 bales. Time to bale and wrap each bale was recorded. Before bales were moved, 4 diameter measurements were recorded for each end of the bale and 4 length measurements were recorded on each side. The diameter and length measurements were averaged, respectively, to calculate bale volume. Bales were weighed individually and stored uncovered in rows for 5 to 6 months. Bale measurements and weights were recorded again after storage.

Core samples were obtained from each bale at baling and after storage. Ten core samples were obtained randomly from the sides of each bale before and after storage using an 18-inch hay probe. Core samples were split into sections representing the outer 6 inches of the core, and the inner 6 to 18 inches of the core. Core depth sections from 2 bales of the same treatment at each farm were composited into one sample for analysis. All samples were analyzed for chemical composition using near-infrared spectroscopy.

After post-storage core samples and measurements were obtained, bales were subjected to different particle size reduction strategies based upon baling treatment. The NORM bales were ground individually in a tub grinder with a 4- to 6-inch screen and ground hay was deposited directly into a twin-screw mixer wagon (NORM-GRIND; XLRation 2580, Helm Welding Limited, Lucknow, Ontario). Time to grind the entire bale was recorded. The CUT bales were individually placed in the mixer wagon and mixed until the long particles were approximately the same length as the longest particles for NORM-GRIND (approximately 4 inches; CUT-MIX). Time to reduce the particle size to the desired length in the mixer wagon was recorded and the weight was recorded on the wagon scales. After particle size reduction, the wagon was

used to deposit each bale in a windrow, and subsamples were obtained for nutrient analysis and particle size distributions.

Data were analyzed with mixed models in JMP (SAS Institute, Cary, NC). Grinding time, grinding shrink, and post-processing factors were analyzed as a completely randomized design. Bale dimensions, weight, density, and post-processing factors were analyzed as a split-plot design, while pre-processing nutrient analysis was analyzed as a split-split-plot design with treatment as the whole plot, storage period as the sub-plot, and core depth as the sub-sub-plot. Significance was declared at $P < 0.05$, and tendencies were declared at $P < 0.10$.

Results

Baling and Storage

The CUT increased bale weight and density compared with NORM ($P < 0.001$), and despite an increase in baling time for CUT because of the greater mass ($P = 0.01$), there was no evidence of a difference for baling rate ($P = 0.74$). A minor, yet significant ($P < 0.001$) increase in bale length for CUT may have been due to increased internal pressure after baling. Using a precutting system on a farm can enhance efficiency by decreasing the number of bales produced and decreasing the space needed for storage. It could also decrease the time spent wrapping bales since fewer bales would be produced overall.

There was no evidence of a difference between treatment for crude protein ($P = 0.31$), but CUT increased neutral detergent fiber (NDF), ADF, and lignin compared with NORM ($P < 0.01$). This increase in fiber content, albeit numerically small, also increased undigestible NDF ($P \leq 0.01$). Considering the lack of difference between treatments for CP content, it is unclear why there was an increase in fiber components, and a resulting decrease in digestibility for CUT. We would anticipate that a loss of leaves due to the pre-cutting process would reduce CP with a concomitant increase in fiber components. The hay in this study was relatively lower quality, so leaf loss may not have been noticeable by evaluating crude protein alone.

There was an interaction between core depth and storage time for ADF and lignin ($P < 0.01$) whereby both variables increased more for the outer portions of the bale over the storage period than for inner portions. Additionally, 240-h NDF digestibility decreased more for outer layers than inner layers over the storage period ($P = 0.001$). The NDF content also decreased over the storage period. These data confirm the effects of bale exposure to the elements during the storage period, where moisture contributes to the deterioration of the outer portions of the hay bale, leaching soluble nutrients and decreasing the fiber digestibility.

Bale Processing

The CUT-MIX combination increased the time to reduce particle size, and the processing rate compared with NORM-GRIND ($P < 0.001$). However, the CUT-MIX decreased the processing shrink for total pounds of DM lost and as a percentage of the total bale weight ($P < 0.001$). The reduced shrink of total DM and as a percent of bale weight for CUT-MIX compared with NORM-GRIND has the potential to save producers significant money. Because of the nature of the study design, it is difficult to determine the effects of the baling treatments vs. the processing treatments individually. However, these combinations are indicative of on-farm scenarios in which this technology can be utilized.

Despite the reduction in shrink for CUT-MIX during processing, it increased NDF, ADF, and lignin content compared with NORM-GRIND ($P < 0.001$), which is the opposite of the anticipated results. This also reduced NDF digestibility and increased undigestible NDF ($P < 0.01$). It is not clear why this occurred, but it may be a factor of the differences in particle size distribution between treatments. The CUT-MIX had more particles on the top

screen compared with NORM-GRIND ($P < 0.001$), but the opposite was the case for the shortest particles ($P < 0.001$). The use of tub grinders is much more aggressive, resulting in a finer product. While we cannot separate the effects of the baling treatment and processing method in this study, it appears that the NORM-GRIND created a much finer product that had more flowability and was easier to sample compared with CUT-MIX. We hypothesize that the greater percentage of longer particles for CUT-MIX allowed the finer particles to fall through the windrow matrix when the hay was touched for sampling, thus resulting in the greater particle size on the top screens and increased fiber content overall. The increase in longer particles with CUT-MIX may be advantageous in scenarios where diets with adequate physically effective fiber cannot be achieved.

Conclusions

Producing round alfalfa hay bales using a pre-cutting mechanism increased bale mass and density. The CUT treatment significantly increased the content of NDF, ADF, and lignin compared with NORM, but the magnitude of differences was small. During processing, CUT-MIX decreased DM shrink compared with NORM-GRIND, but it increased fiber content and reduced digestibility of the hay, while altering the particle size distribution. The increase in fiber content after processing may have been influenced by sampling error due to different particle size distribution between treatments. Further work should be conducted to determine the cause of the increased fiber content for CUT bales, and additional options for incorporating CUT bales into total mixed rations should be evaluated.

Table 1. Effects of a pre-cutting hay baler on round alfalfa bale baling efficiency, bale weight, density, dimensions, and shrink over a 5- to 6-month storage period

	Treatment		Storage			*P*-value		
Item	Cut	Normal	Begin	End	SEM	Trt	Storage	Trt × storage
Baling time, seconds	60	54	-	-	3.7	0.01	-	-
Baling rate, lb DM/min	1,361	1,346	-	-	118.5	0.74	-	-
Bale characteristics								
Weight, lb DM	1,279	1,131	1,253	1,158	43.5	<0.001	<0.001	0.14
Length, in	62.9	62.1	62.5	62.5	0.09	<0.001	0.81	0.34
Diameter, in	64.9	64.9	65.4	64.3	1.71	0.74	<0.001	0.59
Volume, ft^3	121	119	122	118	6.2	0.01	<0.001	0.82
Density, lb DM/ft^3	10.6	9.6	10.3	9.9	0.37	<0.001	<0.001	0.10
Storage shrink, lb DM	108	84	-	-	26.2	0.02	-	-
Storage shrink, %	7.9	7.0	-	-	1.82	0.16	-	-

DM = dry matter. Trt = treatment.

Table 2. Effect of a pre-cutting baler, core depth, and storage timepoint on alfalfa bale chemical composition, quality, and digestibility[1]

	Treatment		Core depth		Storage			*P*-value[2]				
Item[1]	Cut	Normal	Inner	Outer	Begin	End	SEM	Trt	Core	Storage	Core × storage	Trt × storage
Bale DM%	89.5	89.5	89.8	89.3	90.5	88.6	1.36	0.96	0.01	0.02	0.06	0.46
Crude protein, %	18.8	19.0	19.0	18.8	18.9	18.9	1.07	0.31	0.10	0.36	0.98	0.70
ADF, %	38.2	37.6	37.4	38.4	37.0	38.8	0.48	<0.01	<0.001	<0.001	<0.01	0.41
NDF, %	45.4	44.8	44.9	45.4	44.4	45.9	0.81	<0.01	0.06	<0.001	0.64	0.64
NDFom, %	41.9	41.3	41.4	41.7	40.7	42.4	0.95	<0.01	0.25	<0.001	0.71	0.61
Lignin, %	8.7	8.5	8.5	8.7	8.4	8.9	0.10	<0.01	<0.001	<0.001	<0.001	0.47
Ash, %	11.1	10.8	10.9	11.1	11.1	10.9	0.38	0.32	0.18	0.39	0.08	0.77
ADICP, % DM	0.8	0.8	0.8	0.9	0.8	0.9	0.04	0.36	<0.001	<0.001	<0.001	0.02
ADICP, % CP	4.5	4.4	4.3	4.6	4.1	4.8	0.21	0.08	<0.001	<0.001	<0.001	0.05
NDFD-30h, % NDF	41.1	41.6	41.4	41.3	41.4	41.3	0.70	0.08	0.53	0.41	0.69	0.47
NDFD-48h, % NDF	47.7	48.5	48.1	48.1	48.3	47.9	0.57	0.08	0.98	0.50	0.43	0.77
NDFD-120h, % NDF	49.2	50.0	49.8	49.4	49.9	49.3	0.50	0.06	0.30	0.26	0.20	0.98
NDFD-240h, % NDF	51.2	51.5	51.6	51.1	50.7	52.0	0.80	0.10	0.10	0.11	0.001	0.89
uNDF-30h, % DM	26.7	26.2	26.3	26.6	26.0	27.0	0.47	0.01	0.08	<0.001	0.60	0.48
uNDF-120h, % DM	23.1	22.4	22.6	23.0	22.2	23.3	0.51	<0.01	0.10	<0.001	0.28	0.87
uNDF-240h, % DM	22.2	21.7	21.7	22.2	21.3	22.6	0.52	0.01	0.03	<0.001	0.01	0.76
TTNDFD, % NDF	31.7	32.3	32.8	31.3	33.5	30.6	0.94	0.08	<0.001	<0.001	<0.001	0.49
RFV	121	124	124	121	126	119	3.0	<0.01	0.01	<0.001	0.18	0.57
RFQ	112	117	115	114	118	111	3.2	0.02	0.27	0.001	0.55	0.70

[1]Determined by near-infrared spectroscopy (Rock River Laboratory, Inc., Watertown, WI).

[2]Interactions for treatment × core and the three-way interaction of main effects all lacked significance and are not reported in this table.

DM = dry matter. Trt = treatment. ADF = acid detergent fiber. NDF = neutral detergent fiber. NDFom = neutral detergent fiber as a % of organic matter. ADICP = acid detergent insoluble crude protein. NDFD = NDF digestibility. uNDFD = undigestible NDF. TTNDFD = total track NDF digestibility. RFV = relative feed value. RFQ = relative forage quality.

Table 3. Effect of the combination of baling and processing methods (CUT-MIX vs. NORM-GRIND) on the chemical composition and particle size distribution of alfalfa hay

	Treatment			
Item	CUT-MIX	NORM-GRIND	SEM	*P*-value
DM, %	91.5	91.1	0.36	0.06
CP, %	19.1	19.6	1.26	0.06
ADF, %	41.5	40.3	0.57	<0.001
NDF, %	48.6	47.0	0.96	<0.001
NDFom, %	45.1	43.3	1.05	<0.001
Lignin, %	9.9	9.4	0.14	<0.001
Ash, %	11.1	11.0	0.38	0.93
ADICP, % DM	1.2	1.1	0.05	<0.01
ADICP, % CP	6.3	5.9	0.39	<0.001
NDFD-30h, % NDF	39.7	41.1	0.92	<0.001
NDFD-48h, % NDF	44.6	47.0	0.62	<0.001
NDFD-120h, % NDF	46.9	48.7	0.98	0.001
NDFD-240h, % NDF	49.0	50.8	1.30	<0.01
uNDF-30h, % DM	29.3	27.7	0.49	<0.001
uNDF-120h, % DM	25.8	24.1	0.93	<0.001
uNDF-240h, % DM	24.8	23.2	1.08	<0.001
TTNDFD, % NDF	28.7	29.7	0.94	0.03
RFV	109	114	3.0	<0.001
RFQ	95	105	3.3	<0.001
Processing time, min/bale	11.0	3.6	1.53	<0.001
Processing rate, lb DM/min	121	408	79.1	<0.001
Processing shrink, lb DM[3]	20.1	73.2	6.24	<0.001
Processing shrink, % DM[3]	1.6	8.1	0.95	<0.001
Particle size[2]				
Screen 1, % total DM	35.1	13.7	1.58	<0.001
Screen 2, % total DM	20.3	19.6	1.14	0.04
Screen 3, % total DM	13.3	16.6	0.65	<0.001
Pan, % total DM	31.2	49.9	1.82	<0.001

[1]Determined by near-infrared spectroscopy (Rock River Laboratory, Inc., Watertown, WI).
[2]Screen sizes were 19 mm, 8 mm, and 4 mm, respectively.
[3]Does not include data from one farm due to issues with operating the tub grinder.
CUT-MIX = precutting during baling followed by mixer wagon processing. NORM-GRIND = no precutting during baling followed by tub grinder processing. DM = dry matter. CP = crude protein. ADF = acid detergent fiber. NDF = neutral detergent fiber. NDFom = neutral detergent fiber as a % of organic matter. ADICP = acid detergent insoluble crude protein. NDFD = NDF digestibility. uNDFD = undigestible NDF. TTNDFD = total track NDF digestibility. RFV = relative feed value. RFQ = relative forage quality.

Association of Horizontal Silo Pad Type, Elevation and Core Depth With Indicators of Silo Ramp Hygiene, Forage Quality, and Digestibility

W.E. Brown, N.M. Bello,[1] and M.J. Brouk

Summary

Horizontal silo piles without walls are constructed using packing equipment to adequately pack the forage for air exclusion. During packing, the equipment uses a ramp of forage to access the top of the pile, potentially introducing soil into the forage when the base of the silo is made of soil. Soil contains microorganisms which may cause malfermentation and pose health risks to livestock. The objective of this study was to assess the association of horizontal silo pad type, elevation, and core depth with indicators related to silage hygiene and nutrient quality. We hypothesized that ash and mineral content, microbiological profile, and fermentation profile in silos with soil pads would be indicative of soil contamination, and that measures of potential contamination would be lesser at higher elevations within the silo. Eleven horizontal silos on 7 farms were sampled in a split-split-plot design, with silo pad type as the whole plot factor, elevation on the ramp as the split-plot factor, and core depth as the split-split-plot factor; data were analyzed using mixed models to appropriately recognize experimental units for each factor. Regardless of core depth and elevation, silage pH was increased in concrete pads relative to soil pads. Also, for soil pads, phosphorus (P) was increased in samples of the outer core depth compared to inner core depths. Further, on both pad types, iron (Fe) content was greater at lower vs. medium elevations, but there was no evidence of difference for peak Fe content compared with the other elevations. On soil pads, outer layers had decreased 120- and 240-hour neutral detergent fiber (NDF) and 7-hour starch digestibility compared with inner cores regardless of elevation. The outer segments also had increased pH and decreased density compared with inner core depths, regardless of pad type or elevation. Further, independent of pad type or elevation, outer layers increased NDF, acid detergent fiber (ADF), lignin, ash, and minerals, but decreased crude protein (CP) compared with inner core depths. Additionally, compared with inner layers, outer layers had decreased NDF and starch digestibility, and increased undigestible NDF regardless of pad type or elevation. Overall, changes in Fe and P may be indicative of soil contamination on soil pads. Furthermore, the decreased quality of forage in the outer layers of the silo reinforces the importance of an anaerobic environment for the adequate preservation of silage.

Introduction

Achieving high-quality silage on beef and dairy operations requires careful attention to oxygen exclusion to accomplish excellent fermentation. Silage that is inadequately fermented or allowed to spoil during feed-out can develop secondary aerobic fermentation, which increases dry matter loss, reduces dry matter intake and performance by livestock, and may contribute to animal health issues. Specifically, a lack of pH reduction by lactic acid bacteria at the onset of fermentation or in spoiled sections may allow the growth of *Clostridium spp.*, known to have deleterious effects.

Clostridia are ubiquitous, being found in soil, forage, manure, feed, and milk. The microorganisms multiply when they form spores during less-than-ideal growing conditions that re-emerge when the conditions improve. Some evidence suggests that the prevalence of

[1] Department of Statistics, College of Arts and Sciences, Kansas State University.

clostridia in silage may be influenced by the application of animal manure and the timing of its application. While some authors have eluded that soil contamination may be the primary cause of *Clostridium spp*. contamination in silage due to their ubiquitous nature in soil, a paucity of work has been conducted to quantify contamination of soil in silage. One group monitored radiocaesium and titanium in fresh forages, soil, and silage over time, presenting evidence that soil contamination did occur between harvest and ensiling. One case study identified an extreme case of sand contamination in a silage pile stored on soil as a causative factor for bovine mortality from rumen sand impaction, thus highlighting the possibility of soil contamination during packing by inexperienced individuals. Content of ash and Fe has been utilized as a measure of soil contamination of silages after flooding events, and for hay during normal harvest conditions utilizing different harvesting methods.

Approximately 90% of U.S. dairy farms feed corn silage, and many large farms utilize drive-over silage piles of some type to store large quantities of forage. Drive-over silage piles may have a base of soil, or a harder base such as concrete or asphalt. Therefore, considering the ubiquitous nature of clostridia bacteria in soil, the objective of this study was to assess the association between silage pad type, elevation, and core depth with silage hygiene and nutritional characteristics. We hypothesized that ash and mineral content would be greater on silage piles stored on soil compared with concrete, and that those piles would have greater counts of clostridial bacteria. We also hypothesized that the ash and mineral content would be greater at lower elevations where tires first interact with the ramp.

Experimental Procedures

This observational trial was conducted from November 2019 to March 2020 on 7 farms in north central and western Kansas using forages grown in 2019. All silages had fermented for at least 60 days. In total, 11 drive-over corn, sorghum, and triticale silage piles were sampled, 3 of which were on concrete pads with the remainder on soil pads. Detailed explanation of the silage piles sampled are provided in Table 1. All silage piles were covered with plastic.

Each pile was sampled at 18 locations on the silo ramp where tractors accessed the pile for packing, with cores obtained at an elevation of approximately 2 ft (LOW), 4 ft (MED), and at the peak (PEAK; approximately 18 ft). Specifically, 6 cores were obtained across the ramp at each elevation. Cores of 18 to 22 inches in length were obtained with a coring device 2 inches in width. Cores were separated into two segments, 1) 6 inches of the core representing the outer layers of the silage pile, and 2) the remainder of the core (6 to 22 inches) representing the inner portions of the pile.

After samples were collected, three samples (core triad) from the same median plane, elevation, and core depth were composited for a total of 6 samples for each ramp at each core depth. One half of the silage composite was evaluated for chemical composition by near-infrared spectroscopy (Rock River Laboratory, Inc., Watertown, WI). The remaining half of the composite sample was sent overnight to ARM & HAMMER Animal Nutrition (Waukesha, WI) for microbial analysis and fermentation acid profile.

The study was conducted using a split-split-plot design in a randomized block design with subsampling, with farm as an overarching blocking structure, silo pad type as the whole plot factor, silo as the whole plot, elevation as the split-plot factor, and core depth as the split-split-plot factor. A general linear mixed model was utilized for analysis of continuous variables using the GLIMMIX procedure in SAS (version 9.4, SAS Institute, Cary, NC). Fixed effects included pad type, core depth, elevation, their 2- and 3-way interactions, and forage type. Random effects included farm, silage pile (identified as the cross-product of farm by pad type), main core triad (identified by the cross-product of farm by pad type by elevation), and core triad nested within a pair of elevation-specific triads (identified by the cross-product of farm by pad type by elevation

by core depth), in order to properly recognize the size of the experimental unit for each factor. Responses were either transformed for variance stabilization, or heterogeneous variances were explicitly specified, as appropriate in each case. Model assumptions were evaluated using externally studentized residuals and were considered to be appropriately met. Outliers were diagnosed using an extremely conservative Bonferroni test on studentized residuals.

Results and Discussion

Contrary to our hypothesis, there was no evidence for any differences between pad types in ash, Fe, or *Clostridia spp*. We noted interactions involving pad type for P whereby samples had increased P for outer vs. inner core depths in piles stored on soil, but there was no evidence of difference between core depths on concrete pads. However, we consider these results inconclusive since differences were not noted for other minerals. For soil pads, P was greater in outer vs. inner core depths ($P = 0.03$; Table 7), but there was no evidence of difference between core depths for concrete pads. This could indicate some soil contamination for soil pads, though these results were not consistent with our findings for ash. Coincidentally, the 120-hour ($P = 0.02$) and 240-hour NDF ($P = 0.04$), and 7-hour starch ($P < 0.001$) digestibility were both reduced in outer layers for soil pads compared with concrete pads (Table 7). A direct mechanism for why this occurred on soil pads but not concrete pads is elusive based upon our data, especially considering the lesser pH of soil pads.

The lack of evidence for differences between pad types on ash and minerals may reflect the care taken by drivers of packing equipment not to incorporate soil into the pile, or variability of deterioration in the outer layers of the silage. The degradation in the outer layer of the silage concentrated the ash component, and variability in degradation between silos may have been greater than any introduction of ash from the soil making it difficult to detect differences. The ability to detect significant differences in ash in the outer layer of these piles may have been easier in fresh silage that had not yet been covered.

Most of the significant effects identified in this experiment were for core depth. In our study, lactic acid was greater ($P < 0.001$) and pH was lesser ($P = 0.01$) in inner vs. outer core depths. The outer layers of the silage are more exposed to oxygen, which promotes aerobic degradation, leading to the decreased lactic acid concentration that we observed, and the concomitant increase in pH in outer layers. Butyric acid was only detectable in 7 of the 124 samples analyzed, which is indicative of high-quality fermentation and lack of clostridial fermentation in the silage pile ($P > 0.35$). Of the 7 samples in which butyric acid was detected, 6 were in the outer core depths.

As expected, the aerobic degradation in the outer layers lead to a reduction in the nutritive quality of the forage (Table 2). Indeed, there was an increase in NDF organic matter (NDFom) ($P = 0.08$), ADF ($P = 0.05$), lignin ($P < 0.001$), and a decrease in CP ($P = 0.01$) in outer cores vs. inner cores. The digestibility of the fiber ($P < 0.01$) and 0-hour starch ($P = 0.01$) also decreased in the outer layers compared with inner layers, resulting in greater undigestible NDF ($P \leq 0.01$) and a marginally significant lesser total tract NDF digestibility ($P = 0.06$). Feeding deteriorated silage to livestock *in vivo* has a marked ability to decrease apparent digestibility. Overall, the degradation of carbohydrate and protein leads to a loss of dry matter and an increase in inorganic substances remaining as ash. Our data demonstrate an increase in ash in the outer layers where degradation was present compared with inner layers, and the minerals potassium, P, magnesium, and Fe increased in those outer vs. inner depths, regardless of pad type or elevation along the silage ramp.

In this study, only yeast and clostridia were detectable in sufficient samples to warrant analysis. There was no evidence of any differences between pad type, core depth, or elevation in the probability of finding yeast (Table 5) nor for the quantification of *Clostridia spp.* (Table 4).

However, of those samples on which yeast were detected, greater concentrations of yeast were found in the outer layer relative to the inner layer, though only at medium elevations of the silage ramp (Table 9). An increase in yeasts and molds in the outer layers of the silage pile is common, but differences in enumeration of *Clostridia spp.* is generally mixed.

Conclusions

There were minor indications that soil contamination may have occurred based on the increased mineral content of Fe at low elevations, and for P in outer layers in soil pads. Further, soil pads reduced 120- and 240-hour NDF and 7-hour starch digestibility for outer layers. The most numerous effects noted in this study were for core depth, where outer layers exhibited decreased density, nutritive quality, and nutrient digestibility. Future work should incorporate a more balanced experimental design to increase statistical power and should evaluate single forage types with high buffering capacity that may be more prone to malfermentation from soil bacterial contamination, such as alfalfa or winter forage crops. Further, efforts to secure samples prior to fermentation and degradation of outer layers may enhance the ability to detect subtle differences in ash and mineral content as indicators of soil contamination.

Table 1. Description of horizontal silo forage type and pad type by farm

Item	Pad type	Forage
Farm 1		
Silo 1	Soil	Corn
Silo 2	Soil	Corn
Farm 2		
Silo 1	Soil	Sorghum
Farm 3		
Silo 1	Soil	Triticale
Silo 2	Concrete	Sorghum
Farm 4		
Silo 1	Concrete	Sorghum
Farm 5		
Silo 1	Soil	Triticale
Silo 2	Concrete	Corn
Farm 6		
Silo 1	Soil	Corn/Sorghum
Farm 7		
Silo 1	Soil	Corn
Silo 2	Soil	Corn

Table 2. Association of pad type, elevation, and core depth with the chemical composition (as determined by NIR) and density of silage samples obtained from horizontal silo ramps

	Pad[1]		Elevation[1]			Core depth[1]		*P*-value[2]					
Item[1]	Conc	Soil	Low	Med	Peak	Inner	Outer	Pad	Elev	Core	Pad × elev	Pad × core	Elev × core
DM, %	38.1	34.5	36.4	36.5	36.0	36.0	36.6	0.55	0.89	0.55	0.99	0.28	0.63
	±0.13	±0.07	±0.08	±0.08	±0.08	±0.07	±0.07						
pH	5.0	4.8	4.8	4.8	4.9	4.8	4.9	0.02	0.45	0.01	0.74	0.45	0.43
	±0.10	±0.10	±0.10	±0.10	±0.10	±0.10	±0.10						
CP, %	9.6	10.4	10.0	9.9	10.1	9.9	10.1	0.16	0.56	0.01	0.27	0.21	0.11
	±0.41	±0.29	±0.31	±0.31	±0.31	±0.30	±0.30						
aNDF, %	47.5	46.4	46.8	46.9	47.1	46.5	47.4	0.62	0.92	0.08	0.95	0.88	0.90
	±1.64	±0.94	±1.00	±1.00	±1.00	±0.98	±0.97						
aNDFom, %	43.8	43.0	43.3	43.5	43.5	43.0	43.8	0.91	0.92	0.12	0.98	0.47	0.86
	±5.03	±3.56	±3.10	±3.10	±3.10	±3.09	±3.09						
ADF, %	33.3	32.5	32.9	32.8	33.0	32.5	33.3	0.56	0.88	0.05	0.45	0.78	0.99
	±1.14	±0.64	±0.71	±0.71	±0.71	±0.68	±0.68						
Lignin, %[4]	5.2	5.2	5.2	5.1	5.3	5.0	5.4	0.87	0.76	<0.001	0.24	0.37	0.39
	[4.3, 6.3]	[4.7, 5.9]	[4.8, 5.7]	[4.7, 5.6]	[4.9, 5.7]	[4.7, 5.4]	[5.1, 5.8]						
Starch, %	13.3	13.8	13.3	13.6	13.6	13.6	13.5	0.86	0.91	0.92	0.77	0.78	0.71
	±2.03	±1.14	±1.23	±1.23	±1.23	±1.19	±1.19						
Ash, %	12.6	10.7	11.8	11.1	11.9	10.9	12.3	0.48	0.12	<0.001	0.23	0.35	0.33
	±0.17	±0.10	±0.10	±0.10	±0.11	±0.10	±0.10						
K, %	2.4	2.1	2.3	2.2	2.3	2.2	2.3	0.45	0.64	0.001	0.51	0.41	0.56
	±0.23	±0.14	±0.14	±0.14	±0.14	±0.14	±0.14						
P, %[5,6]	-	-	-	-	-	-	-	0.42	0.14	0.04	0.01	0.03	0.13

continued

Table 2. Association of pad type, elevation, and core depth with the chemical composition (as determined by NIR) and density of silage samples obtained from horizontal silo ramps

	Pad[1]		Elevation[1]			Core depth[1]		*P*-value[2]					
Item[1]	Conc	Soil	Low	Med	Peak	Inner	Outer	Pad	Elev	Core	Pad × elev	Pad × core	Elev × core
Ca, %[4]	0.45	0.32	0.40	0.37	0.38	0.37	0.40	0.15	0.27	0.01	0.77	0.36	0.85
	[0.27, 0.77]	[0.24, 0.44]	[0.32, 0.49]	[0.30, 0.46]	[0.31, 0.47]	[0.33, 0.49]	[0.32, 0.49]						
Mg, %	0.27	0.27	0.27	0.27	0.27	0.26	0.28	0.98	0.54	<0.001	0.98	0.48	0.55
	±0.03	±0.02	±0.02	±0.02	±0.02	±0.02	±0.02						
Fe, ppm[4]	418	468	518[a]	381[b]	438[ab]	394	496	0.78	0.02	0.01	0.29	0.37	0.23
	[151, 1156]	[262, 835]	[347, 774]	[255, 569]	[293, 656]	[271, 573]	[341, 722]						
NH_3 N, %	0.17	0.17	0.18	0.18	0.17	0.17	0.18	0.69	0.42	0.06	0.65	0.71	0.17
	±0.01	±0.007	±0.008	±0.008	±0.008	±0.008	±0.008						
NH_3 N, % CP[4]	10.2	8.9	9.6	9.9	9.3	9.5	9.6	0.23	0.61	0.93	0.94	0.33	0.28
	[7.8, 13.0]	[7.6, 10.3]	[8.5, 10.8]	[8.8, 11.1]	[7.8, 10.7]	[8.5, 10.7]	[8.5, 10.8]						
ADICP, %	0.85	0.91	0.90	0.87	0.88	0.86	0.90	0.64	0.88	0.19	0.58	0.86	0.99
	±0.097	±0.056	±0.061	±0.061	±0.061	±0.059	±0.059						
ADICP, % CP	8.5	8.7	8.8	8.5	8.5	8.2	9.0	0.85	0.40	<0.01	0.70	0.48	0.88
	±1.07	±0.62	±0.64	±0.64	±0.64	±0.63	±0.63						
Lactic acid, %	2.03	3.37	2.73	2.79	2.57	3.34	2.05	0.40	0.81	<0.001	0.75	0.50	0.86
	±0.86	±0.50	±0.54	±0.54	±0.55	±0.51	±0.53						
Acetic acid, %[5]	-	-	1.80	1.75	1.63	-	-	0.49	0.68	0.30	0.82	0.07	0.82
			±0.37	±0.37	±0.38								
Density, kg DM/m[3,4,7]	243	272	-	-	-	-	-	0.48	0.74	0.05	0.90	0.11	0.08
	[164, 359]	[217, 240]											

[1]Means are reported with ± SEM unless denoted otherwise.
[2]There was no evidence for any 3-way interaction ($P > 0.27$).
[3]Percentage values are represented as % of dry matter (DM) unless otherwise noted.
[4]Means are reported with 95% confidence interval due to transformation of the data.
[5]Estimated means for the group effects by combination of pad type and core depth are reported in Table 7.
[6]Estimated means for the group effects by combination of pad type and elevation are reported in Table 8.
[7]Estimated means for the group effects of elevation and core depth are reported in Table 9.
[a,b]Estimated marginal means for elevation with differing superscripts are different ($P < 0.05$).
NIR = near-infrared spectroscopy. CP = crude protein. aNDF = adjusted neutral detergent fiber. aNDFom = Amylase-treated neutral detergent fiber as a % of organic matter. ADF = acid detergent fiber. K = potassium. P = phosphorus. Ca = calcium. Mg = magnesium. Fe = iron. N = nitrogen. ADICP = acid detergent insoluble crude protein.

Table 3. Association of pad type, elevation, and core depth with the digestibility (as determined by near infrared spectroscopy (NIR)) of silage samples obtained from horizontal silo ramps

	Pad[1]		Elevation[1]			Core depth[1]		*P*-value[2,3,4]			
Item	Conc	Soil	Low	Med	Peak	Inner	Outer	Pad	Elev	Core	Pad × core
NDF digestibility											
30 h, % NDF[5]	41.8	44.3	43.3	43.3	42.5	44.3	41.7	0.31	0.55	0.001	0.27
	[35.6, 47.1]	[41.1, 47.3]	[40.7, 45.8]	[41.0, 45.5]	[40.1, 44.8]	[42.2, 46.3]	[39.5, 43.9]				
48 h, % NDF[5]	56.6	57.8	57.4	57.9	56.3	58.2	56.2	0.74	0.36	<0.01	0.38
	[46.3, 65.3]	[52.2, 63.0]	[53.4, 61.1]	[54.0, 61.5]	[52.4, 60.1]	[54.7, 61.6]	[52.5, 59.6]				
120 h, % NDF[5,6]	-	-	60.1	60.9	59.1	-	-	0.79	0.35	<0.01	0.02
			[56.0, 64.0]	[56.8, 64.7]	[55.0, 63.0]						
240 h, % NDF[5,6]	-	-	61.8	63.2	60.9	-	-	0.90	0.19	<0.01	0.04
			[57.1, 66.2]	[58.6, 67.5]	[56.1, 65.3]						
Undigestible NDF											
30 h, % DM	27.6	25.7	26.5	26.4	27.1	25.8	27.6	0.12	0.43	<0.001	0.40
	[25.2, 30.3]	[24.3, 27.3]	[25.3, 27.8]	[25.3, 27.6]	[25.9, 28.3]	[24.8, 26.8]	[26.6, 28.7]				
120 h, % DM	20.5	20.2	20.3	20.0	20.9	19.8	20.9	0.90	0.25	0.01	0.19
	±1.71	±1.04	±1.09	±1.09	±1.09	±1.07	±1.07				
240 h, % DM	19.3	19.1	19.1	18.8	19.7	18.6	19.8	0.94	0.21	0.01	0.28
	±1.72	±1.05	±1.09	±1.09	±1.09	±1.07	±1.07				
TTNDFD, % NDF[7]	36.1	34.4	35.5	35.8	34.4	35.8	34.7	0.60	0.46	0.06	0.86
	±2.43	±1.44	±1.57	±1.57	±1.56	±1.43	±1.44				
Starch digestibility											
0 h, %	32.5	27.8	29.6	29.1	31.9	32.4	28.0	0.35	0.46	0.01	0.81
	±2.52	±1.48	±2.05	±2.04	±2.01	±1.66	±1.73				
7 h, %[6]	-	-	65.3	65.8	67.9	-	-	0.67	0.18	0.19	<0.001
			±1.86	±1.88	±1.86						

continued

Table 3. Association of pad type, elevation, and core depth with the digestibility (as determined by near infrared spectroscopy (NIR)) of silage samples obtained from horizontal silo ramps

	Pad[1]		Elevation[1]			Core depth[1]		*P*-value[2,3,4]			
Item	Conc	Soil	Low	Med	Peak	Inner	Outer	Pad	Elev	Core	Pad × core
Milk2006 30 hr											
kg milk/mt	1,070	1,085	1,072	1,099	1,061	1,110	1,045	0.74	0.16	<0.001	0.89
	±69.7	±47.0	±33.4	±33.4	±33.4	±30.8	±30.8				
TDN, %	48.2	52.6	50.2	50.9	50.0	51.2	49.6	0.08	0.20	<0.001	0.88
	±1.56	±1.00	±1.04	±1.04	±1.04	±1.02	±1.02				
NE_L, Mcal/kg	1.21	1.20	1.20	1.23	1.20	1.23	1.18	0.75	0.12	<0.001	0.34
	±0.014	±0.009	±0.010	±0.010	±0.010	±0.010	±0.010				
Milk2006 48 hr											
kg milk/mt	1,182	1,151	1,156	1,186	1,157	1,192	1,140	0.68	0.16	<0.001	0.71
	±88.2	±49.5	±49.7	±49.7	±49.7	±49.6	±49.6				
TDN, %	50.4	54.1	52.0	52.8	51.9	52.9	51.6	0.11	0.19	<0.001	0.81
	±1.50	±1.00	±1.01	±1.01	±1.01	±1.00	±1.00				
NE_L, Mcal/kg	1.23	1.22	1.22	1.24	1.21	1.25	1.20	0.68	0.09	<0.001	0.31
	±0.016	±0.010	±0.011	±0.011	±0.011	±0.010	±0.010				

[1]Estimated means are reported with ± SEM unless otherwise noted.
[2]There was no evidence for any 3-way interaction ($P > 0.17$).
[3]There was no evidence for any 2-way interaction between pad type and elevation ($P > 0.13$).
[4]There was no evidence for any 2-way interaction between elevation and core depth ($P > 0.14$).
[5]Estimated means are reported with the 95% confidence interval after back-transformation of the data.
[6]Estimated means for the group effects of pad type and core depth are reported in Table 7.
[7]Total tract neutral detergent fiber (NDF) digestibility.
NIR = near-infrared spectroscopy. DM = dry matter. TDN = total digestible nutrients. NE = net energy.

Table 4. Association of pad type, elevation (elev), and core depth with the quantification of *Clostridium spp.* and yeast from silage samples obtained on horizontal silo ramps

	Pad[1]		Elevation[1]			Core depth[1]		*P*-value[2]					
Item	Concrete	Soil	Low	Medium	Peak	Inner	Outer	Pad	Elev.	Core	Pad × elev	Pad × core	Elev × core
log_{10} CFU/g[3]													
Clostridia	2.4 ±0.59	2.5 ±0.37	2.5 ±0.31	2.3 ±0.32	2.7 ±0.32	2.4 ±0.30	2.6 ±0.31	0.92	0.23	0.12	0.10	0.11	0.87
Yeast[4]	5.3 ±0.80	5.0 ±0.46	-	-	-	-	-	0.72	0.47	<0.01	0.40	0.64	0.05

[1]Estimated means are reported with ± SEM.

[2]There was no evidence for any 3-way interaction ($P > 0.38$).

[3]Data for quantification of CFU was determined only using data for samples greater than the minimum detectable limit as outlined in Table 5.

[4]Estimated means for the group effects of elevation and core depth are reported in Table 9.

Table 5. Association of pad type, elevation, and core depth on the probability of detection of *Clostridium spp.* and yeast in silage samples obtained from horizontal silo ramps

	Microbe type
Item	Yeast probability (95% CI)
Pad type	
Concrete	0.95 (0.06, 1.00)
Soil	0.76 (0.16, 0.98)
Elevation	
Low	0.91 (0.44, 0.99)
Medium	0.89 (0.38, 0.99)
High	0.86 (0.34, 0.99)
Core depth	
Inner	0.87 (0.43, 0.98)
Outer	0.90 (0.49, 0.99)
P-value	
Pad	0.42
Elevation	0.87
Core depth	0.69
Pad × elevation	0.54
Pad × core	0.69
Elevation × core	0.46
Pad × elevation × core	0.53

Table 6. Empirical prevalence of microbiological species silage samples obtained from the ramp of horizontal silos

Item	Total detected, n[1]
Clostridia spp.	89/124 (0.71)
C. perfringens	44/124 (0.35)
E. coli	29/124 (0.23)
Total coliforms	54/124 (0.45)
Yeast	84/124 (0.68)
Mold	46/124 (0.37)

[1]Proportion of samples in which a given microbe type was detected, and percent of total in the parenthesis.

Table 7. Estimated group-level means for outcomes with marginally or significant interactions between pad type and core depth

	Concrete[1]		Soil[1]		*P*-value
Item	Inner	Outer	Inner	Outer	Pad × core
Phosphorus, %[2]	0.24	0.24	0.25[a]	0.27[b]	0.03
	[0.22, 0.27]	[0.22, 0.27]	[0.23, 0.27]	[0.25, 0.29]	
Acetic acid, %	1.68	1.23	1.94	2.07	0.07
	±0.63	±0.63	±0.36	±0.37	
NDFd 120 h, % NDF[2]	59.8	59.2	62.4[a]	58.7[b]	0.02
	[53.2, 65.7]	[52.6, 65.2]	[58.7, 65.9]	[54.6, 62.4]	
NDFd 240 h, % NDF[2]	62.1	61.3	64.5[a]	59.9[b]	0.04
	[51.7, 71.0]	[50.8, 70.2]	[59.1, 69.6]	[54.0, 65.2]	
Starch digestibles, 7 h, %	64.8	66.6	68.9[a]	64.8[b]	<0.001
	±2.49	±2.49	±1.76	±1.78	

[1]Estimated means are reported with ± SEM unless otherwise noted.
[2]Estimated means are reported with 95% confidence intervals after back-transformation of the data.
[a,b](Adjusted $P < 0.05$) Differences between elevations within a given pad type, either concrete or soil.
NDF = neutral detergent fiber.

Table 8. Estimated group-level means for outcomes with significant interactions between pad type and elevation

	Concrete			Soil			Pad ×
Item	Low	Medium	Peak	Low	Medium	Peak	elevation
Phosphorus, %[1]	0.23[a]	0.24[ab]	0.26[b]	0.26	0.26	0.26	0.01
	[0.20, 0.26]	[0.21, 0.27]	[0.23, 0.30]	[0.24, 0.29]	[0.24, 0.28]	[0.23, 0.28]	

[1]Estimated means are reported with the 95% confidence interval after back-transformation of the data.
[a,b](Adjusted $P < 0.05$) Differences between elevations within a given pad type, either concrete or soil.

Table 9. Estimated group-level means for variables with a marginal or significant interaction between elevation and core depth

	Low		Med		Peak		*P*-value
Item	Inner	Outer	Inner	Outer	Inner	Outer	Elevation × core
Density, kg/m^3[3, 1]	276	249	279[a]	235[b]	247	258	0.08
	[234, 326]	[210, 293]	[236, 329]	[199, 277]	[209, 291]	[218, 304]	
Yeast, $\log_{10}$ CFU/g[2]	5.2	5.2	4.1[a]	5.5[b]	5.1	5.8	0.05
	±0.58	±0.56	±0.58	±0.57	±0.56	±0.58	

[1]Estimated means are presented with the 95% confidence intervals after back-transformation.
[2]Estimated means are presented with ± SEM.
[a,b](Adjusted $P < 0.05$) Differences between core depths within a given elevation, either low, medium, or peak.

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Biological Variability and Chances of Error

Variability among individual animals in an experiment leads to problems in interpreting the results. Although cows on treatment X may have produced more milk than those on treatment Y, variability within treatments may indicate that the differences in production between X and Y were not the direct result of treatment alone. Statistical analysis allows us to calculate the probability that such differences occur because of the treatment applied rather than from chance.

In some of the articles herein, you will see the notation "$P < 0.05$." That means the probability of treatment differences resulting from chance is less than 5%. If two averages are reported to be "significantly different," the probability is less than 5% that the difference is from chance, or the probability exceeds 95% that the difference resulted from the treatment applied.

Some papers report correlations or measures of the relationship among traits. The relationship may be positive (both traits tend to get larger or smaller together) or negative (as one trait gets larger, the other gets smaller). A perfect correlation is one (+1 or -1). If there is no relationship, the correlation is zero.

In other papers, you may see an average given as 2.5 ± 0.1. The 2.5 is the average, 0.1 is the "standard error." The standard error is calculated to be 68% certain that the real average (with an unlimited number of animals) would fall within one standard error from the average, in this case between 2.4 and 2.6.

Using many animals per treatment, replicating treatments several times, and using uniform animals increase the probability of finding real differences when they exist. Statistical analysis allows more valid interpretation of the results, regardless of the number of animals in the experiment. In all the research reported herein, statistical analyses are included to increase the confidence you can place in the results.

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