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Effects of in-feed enzymes on milk production and components, reproduction, and health in dairy cows

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ABSTRACT

Our objectives were to characterize responses in the field to a mix of fibrolytic enzymes using large commercial dairy herds and sufficient study power to evaluate milk production and reproductive responses to an enzyme treatment started during the precalving period. We hypothesized that the use of the enzyme treatment would increase milk production when provided to dairy cows precalving and for approximately 200 d of lactation. The study was conducted on 7,507 cows, in 8 replicates and 16 pens, at 3 dairies in the United States. Eight pens were randomly allocated as control pens and received no enzyme, and another 8 pens received enzyme treatment at a dose of 750 mL/t of dry matter feed. Milk production and energy-corrected milk yield were increased with the enzyme treatment by 0.70 and 0.80 kg/d, respectively, across a 5-month period. Milk fat percentage was not significantly increased by enzyme treatment, but milk fat yield was significantly increased by 0.040 kg/d, compared with controls. Milk protein yield increased 0.010 kg/d with enzyme treatment despite a small reduction of 0.020 percentage units in milk protein percentage. We found no evidence of an increase in the ln somatic cell count for the enzyme-treated cows. Body weight overall was not increased for enzyme-treated cows, but we did observe a numerical increase in dry matter intake (0.20 kg/head per day) for enzyme-treated cows. Most production responses to the enzyme treatment were influenced by dairy. Compared with controls, milk yield in enzyme-treated cows was significantly higher by 3.6 kg/d in dairy 2 and numerically higher by 0.60 and 0.20 kg/d in dairies 1 and 3, respectively. Reproduction, health, and risk of removal or death were not significantly influenced by treatment, apart from a reduced time to first breeding. Production responses to the enzyme treatment varied by dairy from substantial to minor increases, but variation among dairies was not evident in differences in dry matter intake or in partitioning of body weight among enzyme-treated and control pens and cows. It appears likely that the increase in production reflected increased digestibility of feed; however, further work is needed to identify factors influencing the variation in production responses to enzymes.
Key words: fibrolytic enzyme, milk production, reproduction

INTRODUCTION

The specific mix of fibrolytic enzymes applied in this study has been used to increase milk production, as demonstrated in individual studies (Rode et al., 1999; Yang et al., 1999, 2000) and as one of several enzyme products evaluated in meta-analyses (Arriola et al., 2017; Tirado-González et al., 2018). The latter studies indicated that the use of enzymes increased milk and milk protein production. The intervention has been effective in increasing milk and milk solids production, but results are variable, as indicated by significant heterogeneity of responses in the study of Arriola et al. (2017).

Factors that influence responses to enzyme treatment are not well characterized. Inconsistent milk production responses to fibrolytic enzymes, in general, have been attributed to differences in enzyme activity, application rate and composition, dairy cow stage of lactation, mode and time of enzyme delivery, ruminal microbial activity, ruminal enzyme stability, enzyme-feed specificity, and the portion of the diet to which enzymes are applied (Beauchemin et al., 2004; Adesogan, 2005). Additional factors that may explain the variability include insufficient statistical power, inappropriate experimental designs and or durations, inappropriate enzyme choices, and use of inappropriate measures of enzyme activity and misleading enzyme designations (Arriola et al., 2017). Finally, the physical form of application of enzymes may also be an important consideration in understanding variability; moisture may be required to provide effective distribution to sites of action.

The objectives of this study were to characterize responses in the field to a specific mix of fibrolytic enzymes in commercial dairy herds with sufficient study power to evaluate the milk production and reproductive responses to the enzyme treatment that commenced during the precalving period. The hypothesis was that the use of the enzyme treatment would increase milk production when provided to cows precalving and for approximately 200 d of lactation. A strong secondary interest was to evaluate the effects of the enzyme on reproductive performance.

MATERIALS AND METHODS

This study was approved by the University of California, Davis, Animal Care and Use Committee.

Dairy Selection Criteria

We selected 3 dairies that provided a total of 8 replicates with 16 pens (8 enzyme and 8 control) for use in the study, because they had good record keeping and a history of performance that suggested they would be capable of maintaining attention to detail consistent with successful study conduct. Specifically, the dairies met the following criteria: an ability to record daily milk and milk solids production on a regular basis; information available on the cows used in the study to provide clear identification, parity, and pregnancy status; an ability to ensure that feeding was conducted accurately and weights of feed offered and orts could be measured; cows weighed daily or more often; a monitor present to assist with protocol compliance; and pens used were as identical as possible, with a minimum of 2 per dairy. If necessary, a pen rotation was used to establish different replicates and ensure similarity of environmental exposure for pens. Further selection criteria included that records were easily retrieved and could be validated to evaluate anomalies in data; and the herds were willing to comply with a 150-d study minimum to allow an evaluation of reproduction data.

Dairy 1 provided 2 replicates, was located in the Hamlin County, South Dakota, and was positively ventilated with freely available drinking water. Dairy 2 was in Tulare County, California, and also provided 2 replicates. Dairy 3 was in Kerman, California, and provided 4 replicates; the California dairies were freestall sheds with fans and wetters in loafing areas, and drinking water was freely available. We considered that a geographical spread of the dairies would help to improve the external validity of the study. The study was conducted from December 2015 to January 2018. Standard operating procedures were developed to describe the methods of enzyme application, recording of health and reproductive data, and feeding methods for the dairies. Diagnosis of disease was done primarily by dairy staff.

Treatment Pen Allocation

Pens used in the study were randomly assigned to control or enzyme treatments; parity 1 and older pens were allocated separately by the toss of a coin. All cows in the dry pens (8,173 cows) with a projected calving date entered the study and were allocated systematically based on odd and even ear tags that were randomly assigned by the toss of a coin to control or enzyme treatment. We considered blinding of treatments but rejected it because it was important to minimize the risk of error in treatment allocation during feed mixing. Finally, groups were identified based on differences in an additional colored ear button and as having odd- or even-numbered ear tags.

Sample Size Determinations

The power calculations for the study were based on the milk production responses observed in an enzyme meta-analysis by I. J. Lean et al. (unpublished data) and used the rdpower program in Stata version 14.1 (StataCorp LP, College Station, TX), appropriate for determining sample sizes for a 2-level cluster study design. We assumed that the effect size response would be 1 standard deviation greater than the control. This response was consistent with but lower than milk responses observed in an unpublished meta-analysis of responses for the enzyme treatment (I. J. Lean et al., unpublished data). The intra-class correlation was also evaluated between 0.1 and 0.2. For 6 replicates per treatment with pens of 400 cows, an effect size of 0.6, and intra-class correlation of 0.1, the study power was 0.83. More pens and cows were enrolled to allow for assessment of the reproductive and health responses that were of secondary interest.

Study Treatment

All treated pens were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK), in a liquid pre-treatment. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 μmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 μmol of

reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

Pens and cows were administered the enzyme treatment on a DMI basis, as per label directions, at a rate of 750 mL/t DM of feed. The enzyme was mixed with water and administered directly onto the TMR in mixer wagons. To validate the amount of enzyme being used, we undertook a weekly enzyme reconciliation to ensure that the volume of enzyme used was consistent with the anticipated rate of use. Xylanase and cellulase activity were measured using wet chemistry by Enzyme Services and Consultancy, Innovation and Technology Centre (Ystrad Mynach, UK) to identify the presence of active enzymes in TMR samples. Xylanase activity (EC 3.2.1.8) was analyzed using xylazyme AX 60 mg tablets (Megazyme T-XAX200; Megazyme International Ireland Ltd., Bray, Wicklow, Ireland) as a substrate. Cellulase activity (EC 3.2.1.4) was analyzed using Cellazyme-C tablets (Megazyme International Ireland Ltd.) as a substrate. Cows received enzyme-treated feed or control feed for a minimum of 150 d in lactating cow strings and for variable periods before calving.

Feeding and Feed Analysis

Ration samples were collected for each differently formulated TMR—usually precalving, fresh, and high-yield strings—once a week for nutrient analyses. In short, 3 empty feed tubs were placed in feed bunks just before the mixer wagon delivered a load. Tubs (approximately 8–10 kg as-fed TMR per tub) were then collected and the contents mixed on a large clean cement floor. The TMR pile was then quartered, and opposite quarters were mixed and collected into quart-sized zippered plastic bags for nutrient analyses. Samples were frozen at −20°C before submission for laboratory analysis. Details of the TMR fed precalving and during lactation are provided in Tables 1 and 2, respectively.

For dairies 1 and 3, nutrient analysis of the TMR samples was performed at Dairy One Cooperative Inc., Forage Testing Laboratory (Ithaca, NY) according to wet chemistry AOAC International (1999) methods as follows, unless otherwise stated: DM (method 930.15); amylase and sodium sulfite treated NDF (**aNDF**; Ankom Technology method 6 with solutions from Van Soest et al., 1991); aNDF on an organic matter (ash-free) basis (**aNDFom**; aNDF with the addition of an ashing step to remove inorganic materials such as minerals, soil, and sand by burning the fibrous residue at 550°C for 2 h with solutions from Van Soest et al., 1991); CP (method 990.03); soluble protein (Cornell sodium borate–sodium phosphate buffer procedure); adjusted CP (adjusted CP = CP − unavailable protein + 1%);

crude fat by ether extraction (method 2003.05); ash (method 942.05); lignin (Ankom Technology method 9 for acid detergent lignin with solutions from method 973.18); ADF (Ankom Technology method 5 with solutions from method 973.18); acid detergent insoluble CP (ADF residue analyzed using a Leco TruMac N Macro Determinator; Leco Corp., St. Joseph, MI); neutral detergent insoluble CP (Leco TruMac N Macro Determinator); ethanol-soluble carbohydrates (simple sugars; Hall et al., 1999); and starch (YSI 2700 Select Biochemistry Analyzer; YSI Inc., Yellow Springs, OH). The NFC was calculated as NFC = 100 − (NDF + CP + crude fat + ash). The TDN was based on Weiss (1993). The estimated NE_L was based on NRC (1988). The minerals, with the exception of Cl, were analyzed using a Thermo iCAP 6300 inductively coupled plasma radial spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). The Cl ion was analyzed using a Brinkmann Metrohm 716 Titrino titration unit (Riverview, FL).

Analyses for dairy 2 were performed at Analab (Fulton, IL) by wet chemistry according to AOAC International (1999) methods as follows: absolute DM (method 935.29); CP (method 990.03); aNDF (method 2002.04 using α-amylase and sodium sulfite); ADF (method 973.18); soluble protein (Krishnamoorthy et al., 1982); neutral detergent insoluble CP (method 2002.04 without sodium sulfite and method 976.06); acid detergent insoluble CP (methods 973.18 and 976.06); crude fat by ether extraction (method 920.39 with pentane instead of ether); ash (method 942.05); lignin (acid detergent lignin; method 973.18); starch (Smartchem 170 and Smartchem 200 discrete wet chemistry analyzer method GLU-001-A; Unity Scientific, Brookfield, CT); Ca, P, Mg, K, Na, Fe, Zn, Cu, Mn (method 985.01 by inductively coupled plasma spectroscopy); S (method 923.01); and Cl (method 915.01). Simple sugars were measured using near-infrared spectrometry validated equations based on high-performance anion exchange chromatography using pulse anomeric detection (Metrohm, Herisau, Switzerland) of sample extracts that were calibrated using Sigma-Aldrich (St. Louis, MO) standards. The NFC was calculated as NFC = 100 − [(NDF − NDICP) + CP + ash + (crude fat − 1)], where NDICP is neutral detergent insoluble CP. The TDN was based on Van der Honing and Steg (1990). The estimated NE_L was based on NRC (1988). Feed nutrient analysis results are provided in Table 3.

DMI Determination

The DMI was estimated from daily group feed delivery weights from the mixer wagon and recorded using the Feed Supervisor feed management software (Super-

Table 1. Precalving and high-yield lactation diets by dairy

Ingredient (% of DM)	Dairy 1		Dairy 2		Dairy 3	
	Precalving	Lactating	Precalving	Lactating	Precalving	Lactating
Forage:concentrate	75.0	56.0	70.0	28.1	61.5	32.2
AB20 Bentonite[1]	0.41					
Alfalfa hay			30.0	13.0	21.5	3.62
Alfalfa silage	4.71	18				
Almond hulls				5.80	7.26	12.1
AMINOPLUS[2]		1.60				
Blood meal		1.54				
Calcium salts of long-chain fatty acids		0.77				
Canola meal			7.70	15.0	12.6	11.0
Corn gluten				4.60		2.07
Corn ground		12.0				
Corn rolled			17.0	25.0	15.2	23.8
Corn silage	40.2	38.0	40.0	13.0	34.2	16.4
Cottonseed				6.20		
Cracked pita						6.89
Dried distillers grains						9.48
Energy booster 100[3]						0.840
EnerGII[4]				1.40		
Grass hay	30.1					
Haylage						4.31
iLiquid[5] (anionic molasses)			2.80			
Megalac R[6]			0.900			
Mepron[7]		0.07				
Milo				3.70		
Minerals and vitamins lactation[8]		3.13		2.49		1.50
Minerals and vitamins precalving[9]	3.07		1.60		3.45	
Molasses DC 7R137[10]	3.68					
Molasses whey				0.960		
Prilled fatty acids		1.04				
Sodium sesquicarbonate		1.18				
Soybean hulls	1.10	10.2				
Soybean meal	10.0	6.00				
Tallow	0.22	0.150				
Urea				0.200		0.110
Winter forage						3.96
Wheat hay					5.87	3.96
Wheat silage				2.10		
Wet citrus pulp				1.40		
Wet corn distillers	6.36	6.00		5.10		
Yeast	0.140[11]	0.360[12]		0.050[13]		0.030[13]

[1]Phibro Animal Health Corp., Teaneck, NJ.

[2]AGP Ag Processing Inc., Omaha, NE.

[3]Milk Specialties Global Animal Nutrition, Eden Prairie, MN.

[4]Virtis Nutrition LLC, Corcoran, CA.

[5]Innovative Liquids, El Dorado Hills, CA.

[6]Arm and Hammer Animal Nutrition, Princeton, NJ.

[7]Evonik Corp., Kennesaw, GA.

[8]Includes sources for Ca, P, Mg, K, Na, Cl, Mn, Zn, Cu, I, Co, Se, vitamin A, vitamin D, vitamin E to meet NRC (2001) requirements. Dairy 1 contains Rumensin 90 (Elanco, Greenfield, IN) at 200 mg/head per day and dairy 3 at 450.4 mg/head per day.

[9]Includes sources for Ca, P, Mg, K, Na, Cl, Mn, Zn, Cu, I, Co, Se, vitamin A, vitamin D, vitamin E to meet NRC (2001) requirements. Dairy 2 contains Rumensin 90 (Elanco) at approximately 140 mg/head per day and dairy 3 at 217.68 mg/head per day.

[10]Quality Liquid Feeds LLC, Dodgeville, WI. Contains Rumensin (Elanco) at approximately 300 mg/head per day.

[11]Integral A+ (Alltech, Nicholasville, KY).

[12]Cel-Con (Western Yeast Co., Chillicothe, IL), Omnigen AF (Phibro Animal Health Corp.), Integral A+ (Alltech), and Procreatin 7 (Lesaffre, Marcq-en-Barœul, France).

[13]Diamond V XPC (Diamond V, Cedar Rapids, IA).

visor Systems, KS Dairy Consulting Inc., Dresser, WI) for dairy 1 and EZFeed (DHI-Provo, Provo, UT) for dairies 2 and 3. All 3 dairies corrected feed delivered with residual feed. Dry matter intakes were on a per-pen basis and corrected for residual feed. Then, total corrected DMI was divided by the number of cows in

Table 2. Precalving diet nutrient composition by dairy for control and enzyme-treated cows[1,2]

| | Dairy 1 | | | | | | Dairy 2 | | | | | | Dairy 3 | | | | | |
| | Control | | | Enzyme | | | Control | | | Enzyme | | | Control | | | Enzyme | | |
Item[3] (% of DM, unless otherwise specified)	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
DM (%)	19	50.3	3.06	19	49.5	3.06	62	48.7	3.11	62	48.2	2.90	37	59.5	3.03	37	56.8	4.99
CP	19	15.2	1.51	19	15.7	2.10	62	15.6	1.08	62	15.7	1.19	37	16.3	1.19	37	16.2	0.80
Available protein	11	14.9	0.972	11	15.2	1.59							37	15.0	1.22	37	14.7	0.853
ADICP	11	1.00	0.210	11	0.927	0.245	62	1.05	0.0942	62	1.05	0.095	37	1.35	0.348	37	1.44	0.336
Adjusted CP	19	15.2	1.51	19	15.7	2.05							37	16.1	1.23	37	15.9	0.896
Soluble protein (% of CP)	19	46.3	4.38	19	47.2	3.22	62	34.1	4.01	62	34.0	4.37	37	39.4	3.16	37	40.2	3.01
NDICP	11	2.11	0.722	11	1.85	0.242	62	2.16	0.206	62	2.17	0.226	37	2.70	0.519	37	2.52	0.398
ADF	19	28.8	2.99	19	27.8	3.58	62	25.0	2.81	62	25.5	2.89	37	23.5	2.45	37	23.5	2.39
aNDF							62	41.0	4.37	62	41.5	4.60						
aNDFom	11	41.7	3.20	11	40.9	4.80							37	32.1	3.19	37	30.9	3.00
Lignin	19	4.85	0.813	19	4.64	0.845	62	3.60	0.731	62	3.70	0.670	37	5.09	0.918	37	5.30	0.818
NFC	19	27.7	3.15	19	28.1	3.24	62	33.8	3.99	62	33.3	4.00	37	38.7	3.04	37	39.8	2.72
Starch	19	18.0	2.81	19	17.3	2.20	62	17.2	3.57	62	16.6	3.59	37	21.2	4.40	37	21.6	3.25
Simple sugars	11	3.07	1.37	11	3.07	0.815							37	5.57	1.44	37	5.58	1.61
Crude fat	19	3.54	0.506	19	3.56	0.415	62	3.77	0.500	62	3.65	0.453	37	4.72	1.10	37	4.96	1.17
Ash	19	10.6	1.25	19	10.7	1.19	62	8.01	0.468	62	8.03	0.440	37	8.16	0.553	37	8.24	0.614
TDN	19	62.7	3.09	19	63.3	3.05												
NE_L (Mcal/kg of DM)	19	1.45	0.081	19	1.46	0.0791	62	1.60	0.0776	62	1.59	0.0804	37	1.62	0.0759	37	1.63	0.0781
DCAD (mEq/100 g)	10	−22.3	12.4	11	−27.6	14.5	62	4.10	18.0	62	4.18	18.3	37	−15.7	8.75	37	−15.8	10.3
Mineral (%)																		
Ca	11	0.839	0.111	11	0.848	0.157	62	0.934	0.176	62	0.940	0.185	37	1.09	0.182	37	1.11	0.232
P	11	0.381	0.0411	11	0.388	0.0429	62	0.365	0.0309	62	0.365	0.0318	37	0.333	0.458	37	0.327	0.0321
Mg	11	0.469	0.0792	11	0.485	0.0799	62	0.413	0.0890	62	0.411	0.0928	37	0.373	0.0466	37	0.376	0.0579
K	11	1.55	0.250	11	1.53	0.269	62	1.57	0.378	62	1.57	0.383	37	1.27	0.167	37	1.27	0.180
Na	11	0.129	0.0874	11	0.0982	0.0237	62	0.248	0.0621	62	0.249	0.0714	37	0.201	0.0579	37	0.207	0.0572
S	11	0.471	0.118	11	0.514	0.0874	62	0.245	0.0252	62	0.243	0.0253	37	0.369	0.0377	37	0.369	0.041
Cl	11	1.18	0.411	11	1.38	0.299	62	1.12	0.441	62	1.12	0.451	37	1.21	0.150	37	1.21	0.194
Mineral (mg/kg)																		
Fe	11	388	89.9	11	392	96.7	62	769	321	62	782	307	37	297	65.0	37	305	73.2
Zn	11	76.4	11.3	11	75.7	9.27	62	80.2	30.6	62	78.8	31.8	37	98.8	22.3	37	99.3	22.5
Cu	11	16.9	2.70	11	16.9	2.51	62	12.2	5.98	62	12.5	6.45	37	26.3	13.2	37	27.6	18.1
Mn	11	95.2	12.8	11	90.8	12.2	62	72.0	27.3	62	72.1	29.2	37	58.3	9.40	37	58.4	13.2
Mo	11	1.20	0.276	11	1.22	0.218							37	1.60	0.379	37	1.58	0.462

[1]Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK) in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 μmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 μmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

[2]Kolmogorov–Smirnov testing found no differences between control and enzyme and lactating pens in feed composition for any dairy. Note: ash, Fe, Mn, and Mo were not tested.

[3]ADICP = acid detergent insoluble CP; aNDF = amylase and sodium sulfite treated NDF; aNDFom = aNDF corrected for ash residue; NDICP = neutral detergent insoluble CP; NFC for dairies 1 and 3 = 100 − (NDF + CP + crude fat + ash); NFC for dairy 2 = 100 − [(NDF − NDICP) + CP + ash + (crude fat − 1)].

Table 3. Lactation diet nutrient composition by dairy for control and enzyme-treated cows[1,2]

Item[3] (% of DM, unless otherwise specified)	Dairy 1 Control n	Mean	SD	Dairy 1 Enzyme n	Mean	SD	Dairy 2 Control n	Mean	SD	Dairy 2 Enzyme n	Mean	SD	Dairy 3 Control n	Mean	SD	Dairy 3 Enzyme n	Mean	SD
DM (%)	82	46.5	3.62	88	46.5	3.61	92	55.4	2.95	93	55.3	2.11	112	56.7	6.55	114	57.5	4.12
CP	82	16.0	1.42	88	16.2	1.31	92	18.3	0.99	93	18.4	0.746	112	16.7	1.51	114	16.8	0.966
Available protein	41	15.2	1.15	45	15.1	1.10							112	15.0	1.48	114	15.1	1.09
ADICP	41	1.20	0.43	45	1.10	0.425	92	1.00	0.0621	93	1.01	0.0563	112	1.74	0.490	114	1.69	0.356
Adjusted CP	82	15.9	1.41	88	16.1	1.36							112	16.2	1.21	114	16.2	1.14
Soluble protein (% of CP)	82	41.5	6.39	88	40.3	6.30	92	27.1	2.69	93	27.0	2.29	112	32.5	3.45	114	32.6	3.12
NDICP	41	2.58	0.561	45	2.61	0.574	92	2.32	0.128	93	2.33	0.103	112	2.94	0.530	114	2.88	0.526
ADF	82	23.5	2.94	88	23.0	2.58	92	19.5	1.43	93	19.4	1.07	112	21.7	1.80	114	21.6	1.97
aNDF	41	37.0	4.64	43	35.7	4.14	92	32.3	1.78	93	32.1	1.42						
aNDFom	41	33.9	4.03	45	33.1	3.58							112	30.3	2.52	114	30.5	2.68
Lignin	82	3.69	0.716	88	3.56	0.697	92	3.39	0.362	93	3.40	0.378	112	5.37	0.951	114	5.32	0.938
NFC	82	35.8	3.98	88	36.5	3.30	92	38.4	1.49	93	38.4	1.24	112	39.6	2.98	114	39.3	3.05
Starch	82	24.2	3.77	88	24.7	3.08	92	22.7	1.67	93	22.7	1.35	112	23.9	2.65	114	24.0	3.00
Simple sugars	41	2.29	1.23	45	2.15	0.94							112	5.94	1.91	114	5.61	1.47
Crude fat	82	4.46	0.608	88	4.71	0.493	92	5.74	0.534	93	5.76	0.473	112	5.94	0.801	114	6.10	0.65
Ash	82	8.26	0.81	88	8.26	0.70	92	7.63	0.24	93	7.64	0.22	112	7.43	0.63	114	7.38	0.646
TDN	82	70.2	2.78	88	71.1	2.18												
NE_L (Mcal/kg of DM)	82	1.64	0.0750	88	1.66	0.0553	92	1.75	0.0397	93	1.76	0.0299	112	1.70	0.0526	114	1.70	0.0502
DCAD (mEq/100 g)	41	34.6	14.0	45	35.8	7.11	92	18.0	3.00	93	18.6	2.84	112	13.0	4.46	114	12.9	4.73
Mineral (%)																		
Ca	41	0.774	0.107	45	0.754	0.0766	92	1.00	0.114	93	1.01	0.094	112	0.516	0.08	114	0.511	0.077
P	41	0.380	0.0372	45	0.378	0.0361	92	0.436	0.0342	93	0.441	0.0241	112	0.475	0.0503	114	0.468	0.0439
Mg	41	0.430	0.0462	45	0.422	0.0370	92	0.435	0.0456	93	0.441	0.0419	112	0.289	0.252	114	0.288	0.0236
K	41	1.64	0.275	45	1.61	0.278	92	1.29	0.0919	93	1.31	0.0937	112	1.38	0.134	114	1.38	0.152
Na	41	0.426	0.102	45	0.435	0.0630	92	0.382	0.0701	93	0.393	0.0580	112	0.344	0.0668	114	0.338	0.0619
S	41	0.224	0.0599	45	0.214	0.0204	92	0.286	0.0251	93	0.292	0.0209	112	0.297	0.0366	114	0.296	0.0328
Cl	41	0.430	0.200	45	0.395	0.0842	92	0.493	0.0779	93	0.496	0.0744	112	0.669	0.115	114	0.663	0.0956
Mineral (mg/kg)																		
Fe	41	308	42.2	45	308	61.2	92	323	75.8	93	337	100	112	326	71.5	114	321	67.1
Zn	41	78.2	12.7	45	78.4	9.75	92	108	16.5	93	108	13.4	112	77.9	9.70	114	77.9	8.68
Cu	41	17.1	3.60	45	16.3	2.32	92	16.8	2.71	93	17.1	2.10	112	18.2	3.02	114	18.3	2.66
Mn	41	87.8	13.1	45	86.3	10.2	92	92.4	10.1	93	93.6	7.72	112	53.9	5.37	114	53.8	5.10
Mo	41	1.42	0.332	45	1.32	0.312	92	1.26	0.324	93	1.31	0.608	112	1.26	0.324	114	1.31	0.608

[1] Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK) in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 μmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 μmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

[2] Kolmogorov–Smirnov testing found that only crude fat content differed between control and enzyme and enzyme and lactating pens at dairy 1 ($P = 0.05$). No other results approached significance. Note: ash, Fe, Mn, and Mo were not tested.

[3] ADICP = acid detergent insoluble CP; aNDF = amylase and sodium sulfite treated NDF; aNDFom = aNDF corrected for ash residue; NDICP = neutral detergent insoluble CP; NFC for dairies 1 and 3 = 100 − (NDF + CP + crude fat + ash); NFC for dairy 2 = 100 − [(NDF − NDICP) + CP + ash + (crude fat − 1)].

Table 4. Number of cows excluded from the study from each dairy after enrollment by reason

Reason for exclusion	Dairy			Total
	1	2	3	
Aborted	1	30	6	37
Calved before start	86	0	94	180
Calved within 2 wk of end of study	0	7	0	7
Cow unknown	0	0	54	54
Data did not make sense	10	4	0	14
Died before calving	0	5	13	18
Open	1		20	21
Sold before calving	0	13	1	14
Unknown exclusion	1	1	0	2
Unknown or no calving date	27	6	2	35
Wrong study pen after calving	70	70	144	284
Total	196	136	334	666

the pen that day to estimate individual cow DMI. If we found differences between estimates of numbers of cows in pens, data were deleted for that week (deleted n = 3).

Cows Excluded from the Study

Cows were excluded from the study if they calved before enzyme treatments commenced; aborted; were not pregnant; or were sold before calving. Cows that could not be accounted for from the period of enrollment to lactation entry or that were assigned to the wrong pens at calving for more than 3 consecutive wk were also excluded. These exclusions are detailed in Table 4.

General Censoring

Cows that died or were culled were terminated from the milk and BW data at the time of death or decision to remove the cow from the herd. These cows were censored from the survival, reproduction, and health data at that point. Cows that spent more than 3 consecutive wk on the wrong treatment, including being in the hospital pen, were terminated from the milk and BW data at the date of their last weekly milk average in the correct treatment. Survival, health, and reproduction data for these cows were censored 21 d after entry to the wrong treatment.

Milk Production Data

All dairies milked 3 times per day. Milk fat, milk protein, and SCC were recorded monthly at herd recording tests by the Minnesota (dairy 1), Tulare (dairy 2), and Fresno (dairy 3) DHI organizations.

Daily milk production was recorded using milking equipment from the GEA Group (Düsseldorf, Germany;

dairy 1), BouMatic LLC (Madison, WI; dairy 2), and DeLaval (Tumba, Sweden; dairy 3). Pen movements were reported weekly and used to monitor whether cows were in the correct pens or had been transferred to hospital pens.

Milk yield and component data were included in the analyses from cows that had at least 1 herd test and were allocated to the correct pen. Herd test results collected from cows between 0 and 29 DIM were used as herd test 1, subsequent test results at 30-d intervals were used as tests 2 to 5. Energy-corrected milk was calculated as ECM = [(0.3246 × milk yield) + (12.86 × fat yield) + (7.04 × protein yield)], as per NRC (2001).

BW Data

Body weight was recorded on an XR 3000 scale (Tru-Test Limited, Auckland, New Zealand; dairy 1) or an XDS5000 scale (Gallagher Group Pty Ltd., Hamilton, New Zealand; dairies 2 and 3). Body weight was recorded at each milking, but at times the scales failed to record because of physical damage or electrical interference. Some cows had faulty transponders or physical or behavioral attributes that reduced the number of BW measures.

Body weight data were evaluated for implausible results by first excluding weights of more than 1,100 kg or less than 400 kg, and then by removing weights that varied by ±100 kg from one weighing to the next from dairy 1 only. Data were then averaged by cow for each month after calving to produce the final data for evaluation. The removal of weights that varied by ±100 kg from dairy 1 produced similar means and standard deviations to fitting a robust regression model after removal of weights of more than 1,100 kg and using the predicted values derived from this, indicating that the methods used were acceptable.

Disease and Reproductive Events

All cows eligible to enter the study contributed data to the health and reproductive records. Disease and reproductive events including date of calving, breeding events, pregnancy results, hormonal treatments, decisions not to breed, disease diagnosis, disease treatment, and culling were recorded daily using the herd management programs DairyComp305 (Valley Agricultural Software, CA; dairies 1 and 2) or DHI-Plus (DHI-Provo; dairy 3), and exported to Excel and Access (Microsoft Corp.) for processing before statistical analysis.

Health data were entered in accordance with standard operating procedures developed with each dairy. These definitions were largely consistent among dairies and were reviewed for consistency with cow card descriptions and treatments applied.

Cows were both right- and left-censored in the study. Cows that died or were culled before calving were excluded from the reproductive data set because there would have been no intention to breed them. Cows that were designated as "do not breed," including cows identified as being positive for Johne's disease, were censored at the date of that decision. Cows that were not pregnant were censored at the time of removal from enzyme or control pens or at study termination, resulting in full censoring or pregnancy for cows in the study. Most cows had a pregnancy confirmation before removal or after movement to the wrong pen (which allowed determination of pregnancy status at the time of removal), but cows that did not have their status confirmed before removal were considered to be not pregnant at the time of removal. The pattern of censoring was evaluated to identify any possible anomalies in these data.

Statistical Analysis

The unit of interest was the pen in these studies, but the unit of measurement was primarily the cow. Some measures—specifically DMI and diet—were conducted at the pen level.

The feed analysis results were analyzed using a Kolmogorov–Smirnov 2 sample nonparametric test in Stata (version 15.1; StataCorp LLC, College Station, TX) and are provided in Table 3. One DCAD estimate was removed from dairy 1 control pens, because it was an outlier.

Milk data were initially explored for each dairy and evaluated for the normality of distribution of milk production responses (Stata version 15.1; StataCorp LLC). We recognized that cows in later lactations were not highly represented in the data set, and we categorized lactation number into 4 groups; parity or lactation 1, 2, 3, and $\geq$ 4. The following linear mixed model was fitted to the monthly milk production and BW data, because it was consistent with the study design (St-Pierre, 2007):

$$Y_{ijklm} = \mu + \alpha_i + \gamma_j + \theta_k + \delta_{lm} + \beta X_{ijklm} + \omega\alpha\theta\delta\gamma_{ijkl} + f_j + r_{i:nj} + c_{m:ijkln} + \varepsilon_{ijklmn},$$

where μ was the overall mean; α_i was the fixed effect of treatment (i = control or enzyme); γ_j was the fixed effect of dairy (j = dairies 1 to 3); θ_k was the fixed effect of parity (k = parity 1, 2, 3, or $\geq$ 4); δ_{lm} was the fixed effect of time (l = herd test 1 to 5) for cow number (m = 1 to 6,705); βX_{ijklm} was the covariate adjustment for time spent in the pre-fresh pens (days on precalving diet); $\omega\alpha\delta\gamma_{ijkl}$ were the fixed effects of interaction terms, including 2-, 3-, and 4-way interactions of treatment, dairy, parity, and time; f_j was the random effect of dairy; $r_{i:nj}$ was the random effect for the ith treatment within the nth replicate and the jth dairy; $c_{m:ijkln}$ was the random interaction effect associated with the lth herd test of the mth cow with the kth parity within the ith treatment within the nth replicate and the jth dairy; and ε_{ijklmn} was the random error term.

The covariance was unstructured, unless convergence did not occur (milk yield). Marginal means and contrasts were estimated and used to provide estimates of treatment differences. The SCC data were ln-transformed, because these were not normally distributed. Similar approaches were used for DMI, but, enzyme and control pens at the same stage of lactation were the unit of analysis, and these were analyzed using the fixed effects of treatment (enzyme vs. control), dairy (1, 2, or 3), and week, with all 2- and 3- way interactions between them. The data were repeated-measures (week) within experimental units, with the random effects of pen nested within treatment and dairy. The covariance was unstructured. Marginal means and contrasts were estimated and used to provide estimates of treatment differences.

Different time-failure models that accounted for the random effect of identity within treatment, replicate, and dairy were used to assess survival and reproductive outcomes. A comparison of fit for Cox, Exponential, Lognormal, and Weibull models indicated that the Weibull model had the best fit based on Akaike and Bayesian information criteria. These models accounted for the effects of treatment, dairy, and parity and their interactions as fixed, and the random effects of cow identity within treatment, replicate, and dairy. Days on precalving diet was used as a covariate.

Differences in health data were assessed using logistic regression mixed models that accounted for treatment, dairy, and parity for variables with outcomes that were considered dichotomous, and time-failure models for outcomes that reflected time-failure events and accounted for the random effect of identity in treatment, replicate, and dairy and the interactions of the fixed effects of treatment, parity, and dairy. Days on precalving diet were used as a covariate.

RESULTS

The study included 3 dairies and 8 replicates, of which 3 replicates were designated parity 1, providing 16 treatment groups in this study. On an intention-to-treat basis, 8.9% of the cows were lost from the study at the time of enrollment. Most of these were because cows in the dry pen were enrolled before treatments were ready to apply, were not pregnant or were being sold, or were in the wrong pen after calving (3.5% of all cows; Table 4). Of the 18 cows that died before calving, 10 were in the control group and 8 were in the enzyme-treated group. For the health and reproductive data, 7,507 and 7,500 cows were available in the 8 replicates for analysis, respectively.

Diet and Treatment

The diets used are shown in Table 1 and analyzed in Tables 2 and 3. We observed a strong consistency in the delivery of diets; only crude fat content was 0.25 percentage units higher in lactating enzyme-treated pens at dairy 1. Most tested differences for feed components between the enzyme-treated and control pens had $P > 0.700$ for all dairies. Diet differences among dairies were not formally examined in this study, but differences in response to changes in the different feed components are evaluated in a companion paper (H. A. Rossow, H. M. Golder, and I. J. Lean, unpublished).

Assays of the feed for xylanase and cellulase activity and daily monitoring of enzyme use showed that all dairies had enzyme applied to the feed; however, all dairies also had minor lapses in application. In some cases, adequate cellulase activity was present, but xylanase was low, possibly reflecting assay failure.

Production and BW

At herd test, 6,705 cows provided 26,705 observations, except for SCC, which had 493 fewer observations from 6,688 cows. For BW, we had 18,541 readings from 5,828 cows in the 8 replicates. The lower number of cows for BW reflected equipment failures during some periods and a failure of the scale readers to identify some cows.

Table 5. Estimated marginal means ± SE for the effects of enzyme treatment at monthly herd recording[1,2]

Outcome	Mean ± SE		P-value						
	Control	Enzyme	Enzyme (E)	Parity (P)	Dairy (D)	E × P	E × D	E × P × D	Days on pre-calving diet
Milk yield (kg/d)	41.5 ± 0.41	42.2 ± 0.41	<0.001	<0.001	<0.001	0.180	<0.001	<0.001	<0.001
ECM (kg/d)	43.5 ± 0.38	44.3 ± 0.38	<0.001	<0.001	<0.001	0.504	<0.001	<0.001	<0.001
Fat (%)	4.04 ± 0.04	4.05 ± 0.04	0.914	0.072	<0.001	0.499	<0.001	0.782	0.541
Fat yield (kg/d)	1.67 ± 0.02	1.71 ± 0.02	<0.001	<0.001	<0.001	0.429	<0.001	<0.001	<0.001
Protein (%)	2.94 ± 0.02	2.92 ± 0.02	0.008	<0.001	0.898	0.001	0.163	<0.001	<0.001
Protein yield (kg/d)	1.21 ± 0.01	1.22 ± 0.01	<0.001	<0.001	<0.001	0.266	<0.001	<0.001	<0.001
Ln SCC	4.07 ± 0.03	4.07 ± 0.03	0.888	<0.001	<0.001	0.119	<0.001	<0.001	0.012
BW (kg)	623 ± 4.57	625 ± 4.56	0.322	<0.001	<0.001	0.058	0.895	0.605	0.894

[1]Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK) in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 μmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 μmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

[2]Models included the fixed effects of days on precalving diet, enzyme treatment, parity, dairy, herd test and their interactions; and the random effects of identity within treatment and dairy. Herd test was highly significant and not reported.

Cows were fed the control or enzyme-treated diet for an average of 13.8 ± 0.5, 36.9 ± 0.5, and 12.6 ± 0.1 d before calving, at dairies 1, 2, and 3, respectively. Enzyme-treated cows were fed on average 2.4 ± 0.2 d longer than controls; consequently, days on diet precalving was used as a covariate in analyses and was significant for all production measures except milk fat percentage and BW ($P = 0.541$ and 0.894, respectively; Table 5).

Production and BW responses for enzyme-treated and control cows and P-values for effects of enzyme treatment and interactions are provided in Table 5. Estimated marginal means for enzyme-treated and control cows by dairy are shown in Table 6. Figures 1A to H show the marginal means and standard errors plotted over the 5 sampling months for each of the study variables. Milk production and ECM yield were increased by enzyme treatment across the 5-month test period ($P < 0.001$; Table 5; Figures 1A and B, respectively). Milk fat percentage was not significantly affected by enzyme treatment (Figure 1C), but milk fat yield was significantly increased, by 0.040 kg/d (Table 5 and Figure 1D). Although enzyme treatment reduced milk protein percentage by 0.020%, milk protein yield was increased by 0.010 kg/d (Table 5; Figure 1F). We found no evidence of an increase in the ln SCC for the enzyme-treated cows ($P = 0.888$; Table 5; Figure 1G). Overall, BW was numerically 2.0 kg higher for enzyme-treated cows than for control cows ($P = 0.322$; Table 5; Figure 1H). The effect of parity was significant for all production responses except milk fat percentage ($P = 0.072$); the effect of dairy was significant for all except milk protein percentage ($P = 0.898$).

The 3-way interaction between enzyme, parity, and dairy was significant for all production variables except milk fat percentage ($P = 0.163$) and BW ($P = 0.605$). Enzyme by parity was significant only for milk protein percentage ($P < 0.001$). The enzyme × dairy interaction was significant for all production responses ($P < 0.001$) except protein percentage ($P = 0.163$) and BW ($P = 0.895$). For example, compared with controls, milk yield in enzyme-treated cows was significantly higher by 3.6 kg/d at dairy 2 and numerically higher by 0.60 and 0.20 kg/d at dairies 1 and 3, respectively (Table 6). Dairy 2 had greater responses to treatment than dairies 1 and 3, especially in ECM, milk fat, and protein production (Table 6).

DMI

There was a total of 564 weekly observations of DMI across 16 pens. The numerical difference in DMI was 0.20 kg/head per day greater intake for the enzyme-treated group ($P = 0.160$). Dry matter intake for the control and enzyme-treated pens over the 31 wk of the study are shown in Figure 2.
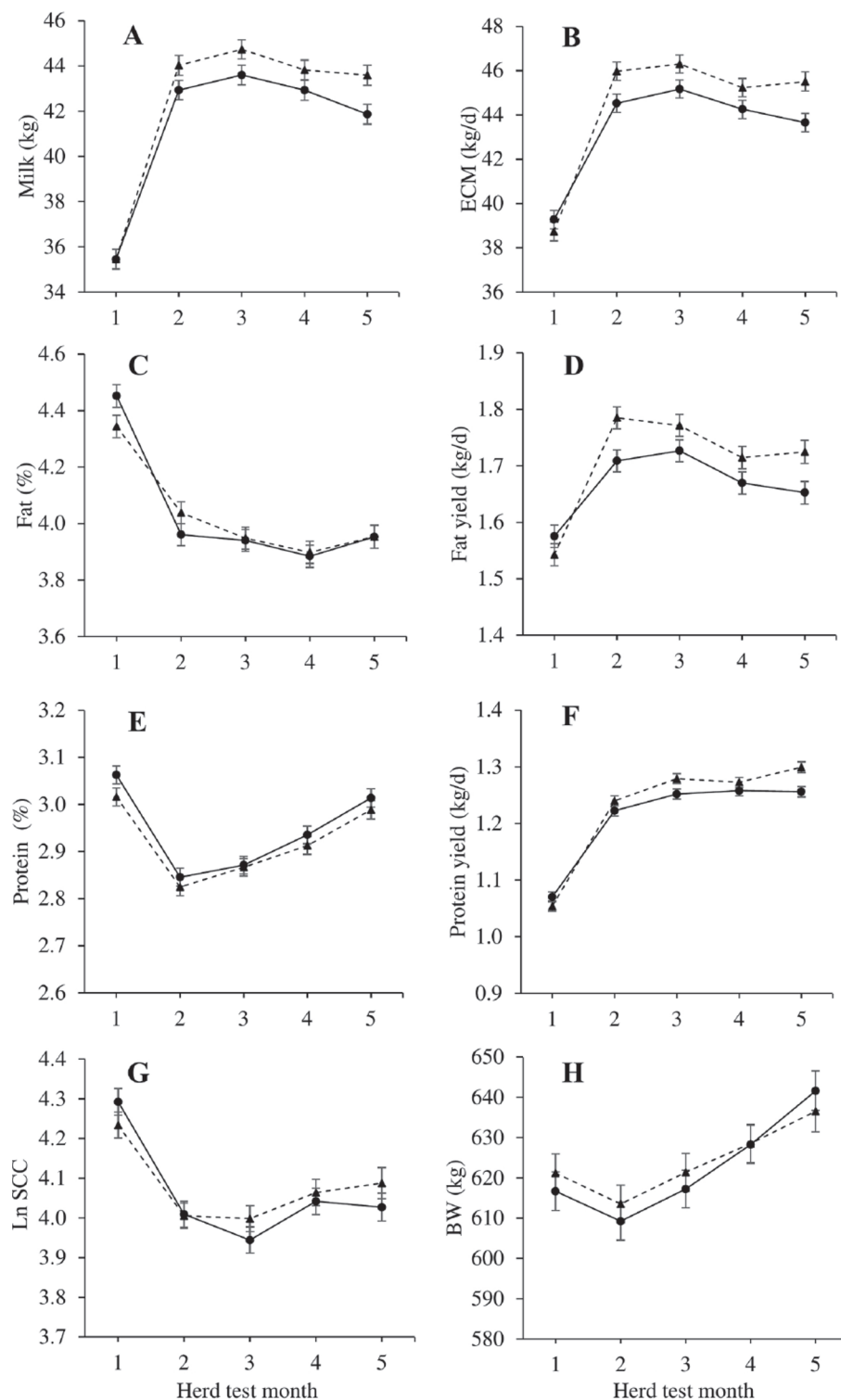
Reproduction and Removal

Figures 3A and B show the cumulative breeding and pregnancy results, respectively, for the 7,500 cows analyzed for breeding or pregnancy using Kaplan–Meier estimates. Figure 3A shows very similar performance for the enzyme-treated and control cows for time to first breeding; however, enzyme-treated cows had a significantly greater probability of being bred per day than the control cows [hazard ratio (**HR**) = 1.266; $P = 0.001$]. However, this effect was influenced by days on the precalving diet (HR = 0.991; $P = 0.001$), dairy ($P < 0.001$), parity ($P < 0.013$), treatment × dairy ($P < 0.001$), treatment × parity ($P < 0.001$), and treatment × dairy × parity ($P < 0.001$). Cows in parity 1 had

Table 6. Estimated marginal means ± SE from monthly herd recording data for the effects of enzyme treatment[1] by dairy

	Dairy 1		Dairy 2		Dairy 3	
Outcome	Control	Enzyme	Control	Enzyme	Control	Enzyme
Milk yield (kg/d)	37.6 ± 0.84	38.2 ± 0.84	37.9 ± 0.82	41.5 ± 0.82	43.5 ± 0.57	43.7 ± 0.57
ECM (kg/d)	42.4 ± 0.76	41.6 ± 0.76	36.8 ± 0.75	41.0 ± 0.76	46.1 ± 0.51	46.2 ± 0.51
Fat (%)	4.56 ± 0.07	4.46 ± 0.07	3.80 ± 0.07	3.86 ± 0.07	4.03 ± 0.05	4.02 ± 0.05
Fat yield (kg/d)	1.73 ± 0.04	1.68 ± 0.04	1.35 ± 0.04	1.54 ± 0.04	1.77 ± 0.03	1.78 ± 0.03
Protein (%)	2.91 ± 0.03	2.92 ± 0.03	2.94 ± 0.03	2.91 ± 0.03	2.95 ± 0.01	2.92 ± 0.01
Protein yield (kg/d)	1.10 ± 0.02	1.09 ± 0.02	1.06 ± 0.02	1.16 ± 0.02	1.29 ± 0.02	1.28 ± 0.02
Ln SCC	4.42 ± 0.06	4.34 ± 0.06	3.68 ± 0.06	3.87 ± 0.06	4.12 ± 0.04	4.09 ± 0.04
BW (kg)	654 ± 9.25	656 ± 9.25	564 ± 9.35	565 ± 9.44	635 ± 6.46	637 ± 6.42

[1]Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK) in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 µmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 µmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

Figure 1. Mean monthly performance ± SEM over 5 herd tests. (A) milk yield, (B) ECM, (C) fat percentage, (D) fat yield, (E) protein percentage, (F) protein yield, (G) ln SCC, and (H) BW for control (●) and enzyme-treated (▲) cows. Herd tests were conducted approximately every 30 d. Herd test results collected from cows between 0 and 29 DIM were used as herd test 1, subsequent test results at 30-d intervals were used for tests 2 to 5. Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK), in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 µmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 µmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.
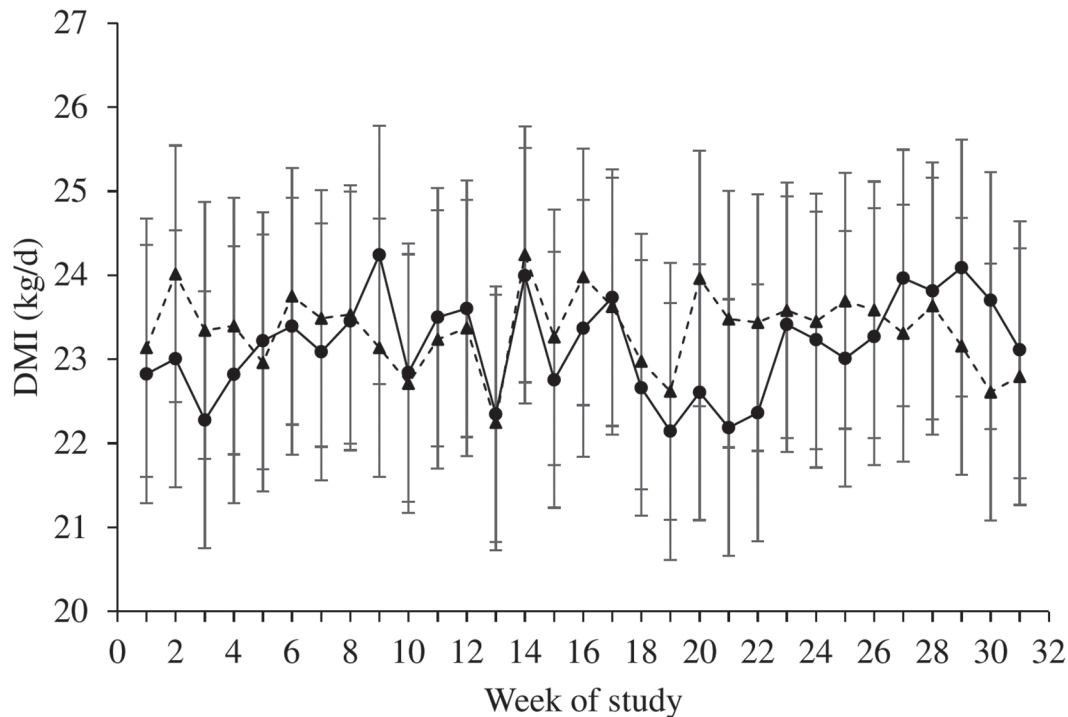
Figure 2. Mean DMI ± SEM from 3 dairies for 8 replicated lactating pens of cows introduced to control (●) or enzyme-treatment (▲) diets before calving by week of study. Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK), in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 μmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 μmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

a lower probability of being bred than cows in parities 2, 3, and ≥4. Compared with dairy 1, cows from dairy 2 had a significantly greater probability being bred each day (HR = 2.064; $P = 0.014$) and cows from dairy 3 had a reduced probability (HR = 0.609; $P = 0.021$). For time to pregnancy (Figure 3B), we found no significant increase in the probability of pregnancy over time (HR = 0.966; $P = 0.949$) for enzyme-treated cows. Days on the precalving diet was not significant (HR = 0.997; $P = 0.066$) and dairy 3 had a significantly lower probability of cows becoming pregnant over time than the other 2 dairies. The interaction of dairy and parity and the 3-way interaction of dairy, parity, and treatment were not significant ($P > 0.100$).

The enzyme-treated cows were not at reduced risk of censoring for pregnancy (HR = 0.907; $P = 0.734$). The interactions of treatment with parity or dairy or their 3-way interaction were not significant ($P > 0.100$). Cows in their fourth parity or more had a markedly greater risk of removal each day than parity 1 cows (HR = 2.21; $P = 0.005$). We found no significant effect of days on the precalving diet (Figure 3B). The removals were not attributable to differences in deaths or culling ($P =$

0.088), as shown in Supplemental Figure S1 (https://doi.org/10.3168/jds.2019-16601). Enzyme-treated cows were 6.2% more likely to stay in the herds over time.

Health

Health on the dairies was assessed using 7,507 cows. The diseases with highest incidence were mastitis, metritis, and lameness. Tables 7 and 8 show the proportion of cows with the different disorders. The only health measure that tended to differ with enzyme treatment was retained placenta, where the odds tended ($P = 0.080$) to be 27% higher in enzyme-treated cows. We found notable differences in the risk or odds of disease for the different parity groups (Tables 7 and 8). Results for the survival analysis models for mastitis and lameness are shown in Table 8. For mastitis, we found a significant ($P = 0.040$) interaction among treatment, parity, and dairy, but none of dairy × treatment ($P = 0.438$), treatment × parity ($P = 0.837$), or the effect of treatment (HR = 1.04; $P = 0.589$) were significant in the survival analysis. Lameness was not significantly increased by treatment (HR = 1.05; $P = 0.875$). The
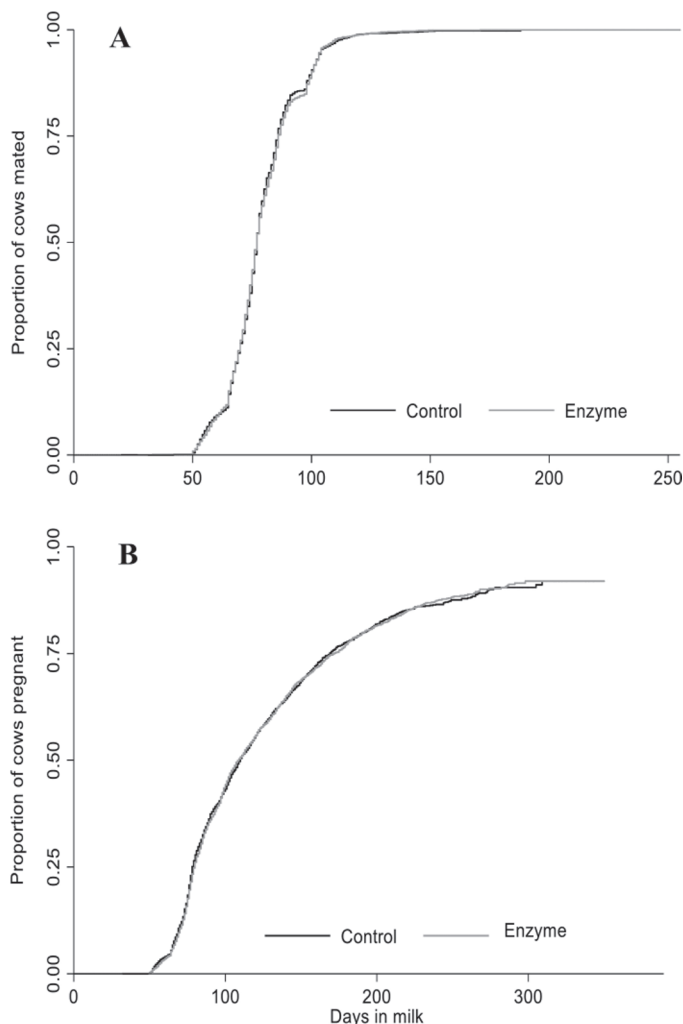
Figure 3. Cumulative proportion of cows in the control and enzyme groups (A) bred by DIM for cows that were bred, and (B) pregnant by DIM for cows that were pregnant. Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK), in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 μmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 μmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

probability of lameness was higher for higher-parity cows than for parity 1 cows, and differed by dairy, but interactions between treatment and parity or dairy were not significant.

DISCUSSION

This work details the responses of cows to an enzyme intervention when the enzyme was introduced to the

Table 7. Proportion of control and enzyme-treated[1] cows with health disorders during the study, odds ratios, and significance[2]

| Disorder | Treatment[3] | | | Parity odds ratio[4] (P-value) | | | Dairy odds ratio[5] (P-value) | | Days on precalving diet odds ratio (P-value) |
	Control	Enzyme	Odds ratio (P-value)	2	3	≥4	2	3	
Retained placenta	0.080	0.092	1.27 (0.083)	1.94 (0.002)	2.00 (0.002)	2.89 (<0.001)	4.43 (0.008)	1.98 (0.148)	0.976 (<0.001)
Pneumonia	0.025	0.027	1.06 (0.734)	1.15 (0.517)	1.79 (0.009)	1.65 (0.052)	0.834 (0.566)	1.19 (0.485)	1.01 (0.104)
Milk fever	0.013	0.009	0.714 (0.151)	23.7 (0.004)	92.5 (<0.001)	343 (<0.001)	2.36 (0.135)	5.94 (<0.001)	1.03 (<0.001)
Metritis	0.228	0.237	1.05 (0.541)	0.384 (<0.001)	0.382 (<0.001)	0.412 (<0.001)	0.325 (0.004)	1.47 (0.235)	1.00 (0.996)
Ketosis	0.026	0.027	0.938 (0.686)	0.708 (0.193)	1.76 (0.020)	2.30 (0.002)	0.124 (0.040)	0.086 (0.006)	1.00 (0.951)
Diarrhea	0.013	0.011	0.819 (0.359)	0.774 (0.527)	2.41 (0.012)	3.25 (0.002)	0.712 (0.493)	0.545 (0.082)	1.00 (0.862)
Displaced abomasum	0.028	0.028	0.903 (0.663)	8.74 (0.002)	21.4 (<0.001)	22.0 (<0.001)	17.3 (0.004)	3.32 (0.153)	1.00 (0.645)

[1]Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK) in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 μmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 μmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

[2]The logistic regression models include the fixed effects of treatment, parity, dairy, and days on precalving diet and the random effects of identity within treatment and dairy.

[3]Reference is control.

[4]Reference is parity 1.

[5]Reference is dairy 1.

Table 8. Proportion of control and enzyme-treated[1] cows with health disorders during the study, hazard ratio, relative risk, and significance[2]

Disorder	Treatment[3]			Parity relative risk[4] (P-value)			Dairy relative risk[5] (P-value)		Days precalving relative risk (P-value)
	Control	Enzyme	Hazard ratio (P-value)	2	3	≥4	2	3	
Mastitis	0.260	0.298	1.04 (0.912)	1.92 (<0.001)	2.98 (<0.001)	3.41 (<0.001)	1.66 (0.017)	4.25 (<0.001)	1.01 (0.016)
Lame	0.115	0.126	1.06 (0.630)	1.06 (0.017)	2.94 (<0.001)	3.75 (0.005)	0.198 (<0.001)	0.605 (0.002)	1.05 (0.875)

[1]Enzyme-treated cows were exposed to a specific mix of fibrolytic enzymes (AB Vista, Marlborough, UK) in a liquid pre-treatment at a rate of 750 mL/t of DM of feed. The enzyme mix is a fermentation product of *Trichoderma reesei* and contains declared minimum activities of 350,000 BXU/g of xylanase (EC 3.2.1.8) and 10,000 ECU/g of cellulase (EC 3.2.1.4), where 1 BXU is the amount of enzyme that will release 0.06 µmol of reducing sugars (xylose equivalents) from birch xylan per minute at pH 5.3 and 50°C; and 1 ECU is the amount of enzyme that will release 0.06 µmol of reducing sugars as glucose from hydroxyethyl cellulose per minute at pH 4.8 and 50°C.

[2]The logistic regression models include the fixed effects of treatment, parity, dairy, and days on precalving diets and the random effects of identity within treatment and dairy.

[3]Reference is control.

[4]Reference is parity 1.

[5]Reference is dairy 1.

diet before calving. The results reflect the responses of a large study involving 3 dairies, 8 replicates, and 7,507 cows. The study design was adapted from those suggested by St-Pierre (2007), because of the strong external study validity achieved by exploring use of the enzyme intervention in the field and the potential to explore any variability in responses by careful monitoring of changes in the diet likely in commercial dairies. Further, this type of study provided opportunities to evaluate the effects of the treatment on reproductive performance and health. Compliance with the protocols for enzyme application was good for the dairies, as indicated by documented use of the enzyme and detection of the xylanase and cellulase activity in feed samples from treated pens. However, despite evidence of enzyme application, optimal xylanase and cellulase activity was not always detected, likely reflecting on-farm mixing failure.

The milk production responses for this study were similar to the results of previous meta-analyses (Arriola et al., 2017; Tirado-Gonazález et al., 2018) showing increased milk production responses to the introduction of an enzyme treatment. Arriola et al. (2017) found that the application of exogenous fibrolytic enzymes increased milk yield by 0.83 kg/d and milk protein by 0.03 kg/d, and found a moderate ($I^2 = 40\%$) level of heterogeneity in milk production responses. This variability was evident in studies that evaluated production responses to the enzyme intervention (Beauchemin et al., 1999; Rode et al., 1999; Yang et al., 1999, 2000), with milk production responses in early-lactation cows of 0.9, 3.6, 1.6, and 2.1 kg/d, respectively,. The cows in our study introduced to enzyme precalving in the transition period responded with increased milk (0.70 kg/d), ECM (0.80 kg/d), milk fat, and milk protein yield. Cows' DMI was not influenced by treatment, and the numeric increase of 0.20 kg/d, measured on a pen basis, did not account for the increase in milk energy production. Cows' BW was numerically 2.0 kg higher for enzyme-treated cows, suggesting that use of the enzyme increased the DM digestibility of the feed. An increase in digestibility of feed is consistent with the increase in digestibility of NDF identified by Arriola et al. (2017). We found marked differences in milk production responses to the enzyme treatment by dairy; the possible reasons for this are explored in detail in a companion paper by H. A. Rossow, H. M. Golder, and I. J. Lean, (unpublished). However, it is evident that diets differed markedly among dairies and this variation, along with the variation in application of the enzyme and environment influenced the responses. Tirado-González et al. (2018) suggests that exogenous fibrolytic enzymes may produce greater production responses when applied to diets that are

higher in forage. In this study, dairy 3 had the lowest NDF concentration in the diet and the lowest response to treatment. Dairy 2, which was the most responsive, had higher concentrations of CP and NDF than dairy 3. Dairy 1 had the highest NDF concentration and a much higher forage-to-concentrate ratio than the other 2 dairies, which provided only about 30% of the diet as forage. Dairy 3 also had the lowest Ca, Mg, and DCAD of the dairies. Further, cows from dairy 3 were also the highest producing, suggesting that their production environment, including nutrition or genetics, was superior to the other dairies. It may be possible that responses to the enzyme did not provide additional nutrients required for increased production at that dairy, or that changes in rumen function resulting from treatment were not favorable; increased fiber digestibility in a herd with low NDF concentration could result in uncoupled fermentation and energy spilling (Strobel and Russell, 1986; Russell, 2007).

The effects of enzyme treatment on reproductive performance and health have not been explored in studies with sufficient power to evaluate these rigorously. We found no difference in time to pregnancy, but time to first breeding was significantly shorter for enzyme-treated cows. However, the latter effect was influenced by significant interactions of treatment with parity and dairy and resulted in very similar performance overall. Although enzyme treatment provided more nutrients for production as evidenced by increased milk and milk solids production, it did not substantially influence reproductive performance. This outcome is similar to that of monensin interventions, which did not alter reproductive performance when the trials of this intervention were evaluated in a meta-analysis (Duffield et al., 2008).

We found no difference in patterns of survival: enzyme-treated cows were more likely to stay in the study, but had a nonsignificantly greater risk of culling and death. The findings in regard to survival are reflected in the health performance of the cows. We found no increase in the probability of mastitis over time, which was 4.0% higher in enzyme-treated cows. The odds of retained placenta tended to be higher (27%) in the enzyme-treated group, but otherwise disease rates were similar between treatment groups. The effects of parity on risk of disease are of interest, because older cows, particularly those in the immediate periparturient period, are at much higher risk of disease.

CONCLUSIONS

The introduction of enzyme treatment to pens of cows in transition and enzyme treatment during the lacta-tion period increased yields of milk, fat, and protein, and reduced time to first breeding, but did not alter time to pregnancy. We found no differences for survival, disease, or ln SCC. Milk yield, ECM, and milk protein and fat production were increased, but the numeric increase in DMI was not sufficient to explain the increase in milk production. This observation combined, with a numeric increase in BW, suggests that treatment increased feed digestibility. There were differences in response to the enzyme treatment among dairies; the sources of variability in response to exogenous fibrolytic enzymes require further evaluation.

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